

STEROID REGULATION OF NEUROPEPTIDES IN SENSORY NEURONS

by

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I declare that the studies presented in this thesis are the result of my own independent investigation unless otherwise indicated in the text.

This work has not been, and is not being, concurrently submitted for any other degree.

ABSTRACT

Primary afferent neurons with perikarya in the dorsal root ganglia (*drg*) mediate the transmission of sensory information from the periphery and viscera to the spinal cord. A number of neuropeptides, including substance P (SP), somatostatin (SS) and calcitonin gene related peptide (CGRP) have been localised in discrete but often overlapping subpopulations of *drg* neurons. These neuropeptides have been implicated in nociception and neurogenic inflammation, both processes which can be influenced by steroid hormones. Whilst steroid hormones have been shown to regulate synthesis of a number of neuropeptides including SP, SS and CGRP in the central nervous system, it is not known if they influence neuropeptides in the *drg*. In this thesis I have investigated the regulation of SP, SS and CGRP by steroids *in vivo* and in dissociated primary cultures of adult *drg* neurons.

The SP, SS and CGRP content of *drg* from C1 to L6 was determined and a differential distribution of the three neuropeptides was found. *Drg* wet weight and SP content were greatest in *drg* from the cervical (C6 - T1) and lumbar (L4 - L6) enlargements. CGRP content tended to be greater in *drg* from more caudal areas (T10 - L6) and SS content was uniform in most *drg* but was notably lower in *drg* from area C5 - C7.

The regulation of neuropeptides in the *drg* by two classes of steroid, adrenal steroids and androgens was investigated. Adrenalectomy (ADX) of adult male Wistar rats increased SP and CGRP and decreased SS content of cervical *drg*. The effects of ADX were reversed by subcutaneous implantation of pellets containing corticosterone (B) or by daily subcutaneous injection of dexamethasone (DEX). The neuropeptide content of *drg* was not regulated by androgens; no significant change in the SP, SS or CGRP contents of *drg* were found following castration or administration of supraphysiological testosterone.

The regulation of neuropeptides in the *drg* by corticosteroids was also examined in a rat model of inflammatory disease. Fifteen days following unilateral inoculation of the carpal joint with a low dose of Freund's complete adjuvant an inflammation of the joint resulted accompanied by a significant increase in the CGRP content of C6/7 *drg* on the ipsilateral side of the animal. SP content of ipsilateral C6/7 *drg* was also increased compared with that of contralateral *drg*, although this did not reach significance: SS content of ipsilateral C6/7 *drg* was not significantly different from that of the contralateral *drg*. ADX of animals suffering from adjuvant induced disease exacerbated and prolonged both the swelling of the ipsilateral forepaw and increased the CGRP content of ipsilateral C6/7 and T1/2 *drg* at 20 days following induction of inflammation.

The effects of corticosteroids on neuropeptides were probably due to a direct action on the *drg*; DEX or B significantly reduced the SP and CGRP contents of cultures of dissociated *drg* grown in the presence of NGF .

The results presented in this thesis indicate that glucocorticoids differentially regulate neuropeptides in the *drg* by a direct action. The therapeutic actions of glucocorticoids may be mediated in part through regulation of neuropeptides in the *drg*.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 THE DORSAL ROOT GANGLION

1.1.1 Introduction

The dorsal root ganglion (*drg*) comprises a collection of neurons and satellite cells contained within a highly vascularised connective tissue compartment. They lie in close proximity to the spinal cord within or near to gaps in the vertebrae found below the transverse processes. The neurons of *drg* develop from progenitor cells of the neural crest, a transient structure overlying the neural tube of the vertebrate embryo which gives rise to a variety of cell tissues and types. Other derivatives of the neural crest include neurons of autonomic ganglia, glial cells, melanocytes and cells of the adrenal medulla. Trunk neural crest cells which migrate ventromedially develop into spinal *drg*, and evidence suggests that the cranial neural crest, in conjunction with placode ectoderm develops into cranial sensory ganglia (Yip 1986).

In their earliest stages of development the ganglionic rudiments comprise a closely packed group of mitotically active cells devoid of blood vessels and connective tissue elements, which appear later in development (Pannese, 1968). The embryonic ganglia grow rapidly through cell proliferation and incorporation of additional migrating neural crest cells (Sobkowicz et al., 1973) and whilst initially, the ventrolateral portion differentiates more rapidly than the dorsomedial, particularly in the avian embryo (Gaik, 1973), this distinction does not persist. In rat, the final division occurs between days 11 and 15 of gestation, with the majority of the large light cells produced on day 12 and the small dark cells on day 13 (see below 1.1.3; Lawson et al., 1974).

The *drg* next undergo a period of post-mitotic maturation during which they extend processes and enlarge. Post-mitotic neuroblasts have several small irregular processes, which are replaced by two neurites formed during

the first stage of differentiation. This may occur through a process of axial elongation and outgrowth from the two poles of the cell, or the peripherally and centrally directed processes may arise, not as distinct neurites, but from constriction and elongation of perikaryal cytoplasm at a single locus, followed by subsequent peripherally and centrally directed elongation (Tennyson, 1965; Sobkowicz et al., 1973). In any event, the peripherally directed process is thicker than the centrally oriented and grows at a faster rate (Pannese, 1974). Concomitant with this process of axon and dendrite extension is a period of rapid increase in cell body volume (of nearly 25-fold, Sobkowicz et al., 1973) and diameter antecedent to a slower expansion, possibly throughout the lifetime of the animal (Hatai, 1902).

During this phase of cellular remodelling leading to the acquisition of a pseudo-unipolar form, a concurrent change in cell ultrastructure and organelle content occurs. The rough endoplasmic reticulum proliferates and Nissl bodies appear, the number of mitochondria substantially increases, the Golgi apparatus forms and microtubules and filaments proliferate (Tennyson, 1965; Sobkowicz et al., 1973). RNA content also increases in parallel with cell volume until adulthood, and the differentiation of *drg* cells into chemically distinct phenotypes, which may begin as early as after the final neuroblast division, continues (Tennyson & Brzin, 1970; Sarratt, 1970; Pannese, 1974).

The satellite and Schwann cells, derived from the neural crest (and possibly neural tube), are initially stellate, appear in the *drg* rudiments after the neuroblasts and elaborate processes to invest a number of neuroblasts (Pannese, 1972). Subsequently, they assume a simpler form, and a more intimate relationship is established in which each satellite cell invests only one neuron. In the early post-natal period, during which considerable enlargement of *drg* cell bodies occurs, the number of satellite cells

undergoes a proportionate increase, and in adulthood, satellite cells retain their mitotic capability (Friede & Johnstone, 1967).

1.1.2 Axons of *Drg*

Kolliker (1844; cited in Lieberman, 1976) first recognised the pseudo-unipolar character of sensory ganglion cells in amphibia and in general, ganglion cells of higher vertebrates are also pseudo-unipolar although in fish, spinal ganglia are bipolar (Chase 1909). The cells give rise to a single principal process, the main process or initial tract of the axon which splits at a T or Y-shaped bifurcation a variable distance from the perikaryon. Whilst the peripherally and centrally directed processes and the stem process that connects them to the cell body are structurally axons and all three may be myelinated, the peripheral process is often referred to as the dendrite and the stem process as a dendro-axonal process (e.g. Warwick & Williams, 197). However, the term dendrite should be reserved for those regions of neurons in which responses are generated and thus strictly applies only to the receptor terminals, found in the periphery (Bodian, 1962).

Afferent fibres conveying impulses from peripheral receptors can be classified as being either non-myelinated or myelinated (depending on the composition of the surrounding Schwann cells). A more extensive functional classification of afferent fibres is also possible based on analyses of fibre diameter and conduction velocity. Originally, this work was performed in frog sciatic nerve by Erlanger & Gasser (1937) who devised the classification A, B and C. Group A comprises the largest fibres with the fastest conduction velocity i.e., myelinated somatic afferent and efferent fibres; group B contains myelinated preganglionic fibres of the autonomic nervous system, and group C is composed of the smallest diameter, slowest-conducting unmyelinated visceral and somatic afferents and post-ganglionic autonomic efferents. Subsequently, Group A has been further subdivided

into A α , A β , A γ and A δ as a result of further combined histological and electrophysiological studies. Table 1.1 gives a description of the classification of afferent fibres from the *drg*, their diameter, and conduction velocity, and the structures they innervate in the cat (Carpenter, 1990).

A number of well-established ultrastructural differences between central and peripheral process of mature and developing neurons have been found. The majority of these are thought to reflect differing transport requirements of central and peripheral processes. Thus, in embryonic and adult avian *drg* cells, a greater number of microtubules are found in peripherally directed processes, and more filaments are present in centrally directed processes of developing (Barasa et al., 1970) and mature neurons (Zenker et al., 1973, 1975). However, some of the differences may not be directly related to axoplasmic transport e.g., the embryonic peripheral process has more granular endoplasmic reticulum and ribosomes than the central (Tennyson, 1965; Zelena, 1972)

Interestingly, axoplasmic flow also differs centrally from peripherally. The rate of fast transport is identical in both central and peripheral processes and to that of a variety of neurons (16mm/h, Lasek, 1970; Ochs, 1972); however, proteins synthesised in the perikarya are transported at a variety of rates along both processes (Droz, 1965; Lasek, 1968) and the net somatofugal flow along the central process is considerably less than in the peripheral process (Lasek, 1968, 1970). Qualitative differences have also been observed; thus, in cat sciatic nerve, electrophoretic analysis of ³H-leucine labelled proteins following injection of tracer into L7 *drg* reveals two peaks of 100K_D and 18K_D in sciatic axons, but in dorsal column axons, only one of 65K_D is found (Anderson & McClure, 1973). It is not clear what these differences in protein labelling reflect. However, it is likely that some centrally transported proteins are concerned with transmitter release in the spinal cord, and peripherally directed proteins may be involved in sensory

Table 1.1 Classification of primary afferent nerve fibres, and the structures innervated by them (Carpenter, 1990)

TABLE 1.1

PROPERTIES OF NERVE IN AXONS

		DIAMETER (μM)	CONDUCTION VELOCITY (m/sec)
A	α	8-20	50-120
	β	5-12	
	γ	2-8	10-50
	δ	1-5	3-30
B		1-3	3-15
C		< 1	< 2 (unmyelinated)

Motor nerve fibres are mostly A- α and A- γ

Skin afferents are mostly A- β , A- δ and C

transduction, trophic interactions with the periphery or antidromic action potentials.

Additional evidence of selective somatofugal transport comes from observations of neurotransmitters in peripherally and centrally directed processes. In dorsal roots, glutamate (a putative primary afferent neurotransmitter) concentrations are higher (and equivalent to that of the perikarya) than in peripheral nerve (Duggan & Johnston, 1970; Johnson & Aprison, 1970); similarly, substance P (SP) is found in higher concentration in peripheral than in central processes (Takahashi & Otsuka, 1975; Harmar & Keen, 1982). Closer examination of the movement of intra-axonal organelles of proximal and distal dorsal roots indicates that saltatory movement of mitochondria and of large vesicles of smooth endoplasmic reticulum occurs in both directions along definite intra-axonal 'tracks' (Smith, 1972, 1973; Cooper & Smith, 1974). Movement is, however, predominantly somatopetal in both proximal and distal fibres, and most particles travelling in both directions are transported at the 'fast' axoplasmic flow rate (see above).

Although it is generally accepted that whilst there is no difference in size between centrally and peripherally directed processes of myelinated axons (Dale, 1900; Rexed & Sourander, 1949), a disparity in the diameters of unmyelinated axons has been acknowledged (Ranson & Davenport, 1931; Ha, 1970). Ultrastructural studies of unmyelinated axons, central and peripheral to *drg*, indicate the mean diameter and range of diameters is considerably less on the central side (Gasser, 1950); this may explain the reported slower conduction velocity of C-fibres on the central side of the *drg* (Mei et al., 1971). It has been suggested, on the basis of ultrastructural studies, that for some neurons, the peripheral process may be myelinated and the central branch unmyelinated (Ha, 1970, 1969). However, only a very small disparity between the numbers of myelinated axons central and

peripheral to *drg* is found (Dale, 1900; Rexed & Sourander, 1949) and it is likely that this form of unequal branching, if it exists, is very rare in mammalian *drg*.

1.1.3 Cell types in the *Drg*

Perikarya of *drg* neurons vary enormously in diameter both between different *drg* and within individual *drg* of adult rat: 20-50 μ m (Hatai, 1901), 10-80 μ m (Cavanaugh, 1951). The neurons are generally spheroidal although some of the smaller cells may be ellipsoidal (Bunge et al., 1967) and are thought to be randomly distributed within the *drg*. (It has been suggested that small cells may be more numerous at the centre and large cells at the periphery of developing human *drg* (McKinnis, 1936) and differences between ventrolateral and dorsomedial cell groups do occur early during development, but only transiently). Cell size is positively correlated with axonal diameter (Cajal, 1909; Lee et al., 1986), the body size of the animal (the largest perikarya are found in the *drg* of large mammals; Levi, 1908), and the extent of the peripheral field innervated (Ohta et al., 1974).

The number of neurons in individual *drg* also varies; thus, in rat, cervical *drg* contain approximately 11,000 cells whereas thoracic ganglia contain only 7,000 cells (Hatai, 1902). In cat, *drg* from C₂-T₁ contain 18-33,000 cells, whereas those from T₃-L₃ contain 8-12,000 cells (Holmes & Davenport, 1940). *Drg* from the cat have also been found to comprise cells of different sizes depending on the site of origin of the *drg* (Larnicol et al., 1988; Rose et al., 1990). Marked differences in cell number between corresponding *drg* on opposite sides of the body have also been reported (Delorenzi, 1937; Ygge et al., 1981).

When examined in the light microscope, the neurons of normal *drg* are usually classified into two main categories: the large light or type A

neuron and the small dark or type B. The small dark cells are more intensely basophilic, argyrophilic and osmiphilic than the large light cells (see Rambourg et al., 1983). A number of other criteria including perikaryal size and morphology and distribution of Nissl bodies have been used to further subdivide neurons (Clark, 1926) into a variety of classifications but with only "... superficial correspondence one with another ..." (Lieberman, 1976). Electron microscopic studies have confirmed the existence of two groups of neurons on the basis of ultrastructural features (Dawson et al., 1955; Jacobs et al., 1975) and histochemical properties (e.g., Tewari & Bourne, 1962; Cauna & Naik, 1963; Kalina & Bubis, 1968; Kalina & Wolman, 1970). Andres (1961) distinguished three subtypes for each of the light (A₁, A₂, A₃) and dark (B₁, B₂, B₃) neurons from the appearance of the Nissl bodies. Duce & Keen (1977) confirmed this configuration and further demonstrated that prolonged nerve stimulation did not modify the relative numbers of the various cell types, suggesting that ultrastructural differences between cell types do not arise as a consequence of their history of activity.

Using improved techniques, Rambourg et al. (1983) further refined and extended Andres' original classification. Compared with Type A₂, Type A₁ cells have narrower strands of cytoplasm separating the Nissl bodies and in addition, contain relatively few neurofilaments whereas A₂ cells have prominent bundles of neurofilaments. A₃ cells are of intermediate size and cytology between B and A type cells; in common with type A cells, they contain Nissl bodies widely dispersed throughout the perikaryon and a well-developed Golgi apparatus which, in combination with mitochondria form a perinuclear ring characteristic of type B cells. Type B₁ cells contain prominent Golgi apparatus surrounding the nucleus forming a broad perinuclear network extending to the nuclear surface and, less commonly, to the cortex of the cell. Type B₂ cells are the smaller of B type cells and exhibit clear cut zonation of their perikaryal organelles. The juxtannuclear

region contains Golgi apparatus and an abundance of mitochondria, whereas the cortical area is occupied by an intricate mesh of rough endoplasmic reticulum. Type B₁ cells are more common than B₂. The Type A cells were the largest (40-75µm in diameter), B cells were of intermediate size (20-50µm) and type C cells were the smallest (less than 20µm) of all ganglion cells with few and small Nissl bodies in the medial zone of the perikaryon and a perinuclear Golgi apparatus. Rambourg's classification is based on ultrastructure and cytology and bears no consideration of a functional classification, however the dark/light distinction (see above) may have some basis in a functional diversity. There is clinical and histological evidence to support the contention that small dark cells are specifically nociceptive (Knyihar, 1971; Gobel, 1974).

In addition, to neurons and satellite cells, *drg* have also been claimed to contain 'atypical' ganglion cells; unipolar cells having, in addition, a number of short dendrite-like processes originating from the cell body and initial tract of the axon. It was initially proposed that 'atypical' cells were a response to injury, but they are present in foetal and neonatal *drg* and it is now generally accepted that they are fixation artefacts due to shrinkage and/or distortion. In the rabbit nodose ganglion, ectopic autonomic neurons can be distinguished in clusters close to the superior pole of the ganglion; they are thought to occur through anomolous development of the vagus, given its close relation to the cervical sympathetic system (see Lieberman, 1976).

Dogiel (1896) and others (see Lieberman, 1976) have described pericellular nests similar to those found in association with autonomic ganglion cells, which Dogiel interpreted as the receptor apparatus of other (sensory) cells within the ganglion. His conclusions have since been confirmed by a variety of workers (reviewed by Milokhin & Reshetnikov, 1972). Cajal (1928) took the view that these pericellular nests were the

terminal arborizations of sympathetic fibres of extrinsic origin, and indeed studies using fluorescence histochemistry do indicate the presence of pericellular skeins of adrenergic fibres (Owman & Santini, 1966; Santini, 1966) around a few cells in cat cerebrospinal ganglia that are reduced following sympathectomy. Despite strong anatomical evidence suggesting a close affiliation of sensory and autonomic cells in the *drg*, they do not appear functionally coupled; no synaptic contact between them can be demonstrated except in dissociated cell culture (Miller et al., 1970; Lodin et al., 1973) and thus, the autonomic ganglion cells in the *drg* may simply be present as a consequence of anomalies during their development.

Electrophysiologically, the perikarya are thought to be of limited functional significance in the intact animal; the perikaryon and initial axon of the ganglion cell are devoid of synaptic contacts and do not normally generate action potentials. Thus, the electrically active parts of the cell are the terminal portion of the peripheral processes and the synaptic endings of the central processes. However, perikarya do maintain a negative resting potential of some 20-90mV and the soma is invaded by action potentials passing along its processes (see Scott et al., 1969). Conduction along the proximal region of the initial section is slow and often the central terminals of large diameter axons are depolarised before the soma (Darian-Smith, 1973). Under experimental conditions *drg* cells can be demonstrated to possess active properties; if cells are fatigued by repeated electrical stimulation of the peripheral nerve, an antidromically directed action potential is sometimes generated by, or close to, the cell body following the electrically induced spike. This antidromic potential is never propagated centrally (orthodromically; Tagini & Camino, 1973) and its significance, if it exists under normal physiological conditions, is unclear. Spontaneously arising impulses can also be detected in isolated *drg* one day or more after isolation, but in this case the impulse was centrally directed (Kirk, 1974).

Another important concern regards the passive delay experienced in the propagation of the action potential as it passes through the *drg*, arising almost certainly from the junction between peripheral and central processes (Dun, 1955).

1.2 NEUROCHEMISTRY OF THE *DRG*

1.2.1 Peptide transmitter candidates of the *drg*

A number of chemically diverse compounds have been proposed to act as neurotransmitters in neurons of the *drg*. Whilst some of the candidates such as SP have been demonstrated within cell bodies by immunohistochemical and other methods and also to be of some functional significance centrally and peripherally, some of the more recently discovered neuroendocrine peptides e.g., corticotrophin releasing factor, localised in *drg* by immunohistochemical methods, have, as yet, no proven function in sensory ganglia and may, in fact, be artefacts due to cross-reactivity. The problem of cross-reactivity encountered using immunohistochemical techniques should not be underestimated, and the suffixes such as 'like immunoreactivity' or 'immunoreactivity' are commonly used when describing peptide localisations. Whilst in this thesis I have chosen, for expediency, to dispense with such terminology, the requirement for caution implied in interpreting results obtained using immunological techniques, including RIA, has not been ignored.

To date, the list of peptides so far discovered includes, SP (Hokfelt et al., 1975; Nagy & Hunt, 1982; Ju et al., 1987), somatostatin (SS, Hokfelt et al., 1975a, 1976; Johansson & Vaalasti, 1987; Ju et al., 1987), calcitonin gene related peptide (CGRP, Rosenfeld et al., 1983; Gibson et al., 1984b; Weisenfeld-Hallin et al., 1984), neurokinin A (NKA, Dalsgaard et al., 1985), neurokinin B (NKB, Too et al., 1989) vasoactive intestinal peptide (VIP, Lundberg et al., 1979; Gibson et al., 1984a), cholecystokinin (CCK,

Lundberg et al., 1978; Ju et al., 1987), mammalian bombesin or gastrin-releasing peptide (Panula et al., 1983; Fuxe et al., 1983), galanin (GAL, Ch'ng et al., 1985; Skofitsch & Jacobowitz, 1985a), oxytocin (OXY), arginine vasopressin (AVP, Kai-Kai et al., 1985, 1986), enkephalin (Senba et al., 1982), dynorphin (Botticelli et al., 1981), endorphin (Itoga et al., 1980), corticotrophin-releasing factor (CRF, Skofitsch et al., 1985), growth hormone-releasing factor (GHRH, Jozsa et al., 1985), endothelin (Giaid et al., 1990), and atrial natriuretic factor (ANF, Nohr et al., 1989).

1.2.1.1 Tachykinins

SP, the most widely studied sensory neurotransmitter candidate, was originally mooted by Lembeck (1953) as a transmitter candidate from the finding that there was greater SP in the dorsal than ventral horn. Later, Angelucci (1956) identified SP as one of a number of bioactive substances released into perfused spinal cord on electrical stimulation of frog hind leg. The suggestion that SP may mediate transmission of nociceptive information to the spinal cord came from the observation that capsaicin, which is known to cause a desensitisation to chemical noxious stimuli, depleted SP from dorsal roots (Gasparovic et al., 1964). SP was also the first sensory transmitter candidate to be localised histologically, to 20% of all cells in a population of predominantly small sized neurons (Hökfelt, 1975a). More recently, preprotachykinin (PPT) mRNA has been localised to the same population of cells as those containing SP (Henken et al., 1988).

SP containing fibres and terminals have been demonstrated in lamina I and II of the dorsal horn (Nilsson et al., 1974; Priestly et al., 1982), and at the resolution of the electron microscope are found to form axo-dendritic contacts with dorsal horn neurons (Chan-Palay & Palay, 1977). Following dorsal root transection or ligation, Takahashi & Otsuka (1975) used bioassay to demonstrate a marked reduction of SP in the dorsal horn,

and these studies have now been replicated by a number of authors using immunohistochemistry or RIA (Hökfelt et al., 1975a; Barber et al., 1979; Jessell et al., 1979).

SP has been demonstrated in a variety of peripheral nerves including phrenic (Malthe-Sørensen & Oktedalon, 1982) and sciatic nerves (Brimijoin et al., 1980; Lundberg et al., 1978; Lembeck et al., 1981), and, following ligation or transection, SP accumulates in the proximal stump and is depleted from the target area; the transport of SP is far greater in the peripheral nerve than the dorsal root (Harmar & Keen, 1982). In the skin, SP-containing fibres were first demonstrated in the hind paw of the cat (Hökfelt et al., 1975); in human skin, they are found as free nerve endings in some Meissner's corpuscles, and in association with blood vessels, sweat glands and hair follicles (Dalsgaard et al., 1983a). SP containing fibres have also been demonstrated in a variety of other tissues (see Dalsgaard, 1988) but of relevance to the role of SP in inflammation, a dense network of SP-containing fibres around blood vessels has been demonstrated (Furness et al., 1982) which is abolished by capsaicin pretreatment (Papka et al., 1984).

Further evidence for a role of SP as a neurotransmitter comes from electrophysiological studies. A peptide extracted from frog dorsal roots, later identified as SP (Takahashi et al., 1974) is known to depolarise frog motoneurons (Otsuka et al., 1972, 1972a). In mammals, a similar effect is also found (Konishi & Otsuka, 1974, 1976) and can be mimicked by capsaicin induced release of SP from the sensory nerve terminals (Theriault et al., 1979; Bucsics & Lembeck, 1981). Furthermore, SP is excitatory on a subpopulation of dorsal horn neurons which are excited by peripheral noxious stimuli (Henry, 1976; Randic & Miletic, 1977).

Other tachykinins have also been found in the *drg*; studies utilising immunohistochemical and radioimmunoassay combined with high

performance liquid chromatography (HPLC) have demonstrated the co-existence of SP, NKA and neuropeptide K (NPK, an N-terminally extended form of NKA) in primary sensory neurons (Minamino et al., 1984a; Dalsgaard et al., 1985; Hua et al., 1985; Ogawa et al., 1985). SP and NKA have similar actions on effector cells (Hua et al., 1985; Nilsson et al., 1985) and are co-released (Saria et al., 1984a). Recently, neurokinin B (NKB) was reported in *drg* in substantially lower concentration than SP or NKA (Too et al., 1989). Thus, from the evidence presented, it seems likely that SP, and possibly NKA in primary sensory neurons, may mediate transmission of nociceptive stimuli.

1.2.1.2 Somatostatin

SS exists in at least two forms (SS-14 and SS-28; see below) and both have been found in primary sensory neurons (Ho & Berelowitz, 1984). SS is present in approximately 10% of all cells, found primarily in small dark neurons (Price, 1985) and SS mRNA has also been localised to these neurons (Henken et al., 1988). In the spinal cord, SS-containing fibres are found in greatest density in lamina II and to a lesser extent in lamina I (Dalsgaard et al., 1981; Nagy et al., 1981); capsaicin pretreatment depletes 20% of SS in the spinal cord (Gamse et al., 1981; Jancso et al., 1981; Nagy et al., 1981) but 90% of SS is depleted from the dorsal roots (Nagy et al., 1981). The presence of numerous SS-containing small cell bodies in the dorsal horn accounts for this difference (Dalsgaard et al., 1981; Ribeiro-da-Silva & Cuello, 1990). SS is transported peripherally, is found in sciatic and splanchnic nerves following ligation (Lundberg et al., 1978; Rasool et al., 1981) and cell bodies retrogradely labelled from cutaneous and muscular nerves are found to contain SS (Molander et al., 1987). In the human skin, free nerve endings contain SS, suggesting a peripheral role for SS in sensory perception (Johansson & Vaalasti, 1987); the presence of SS

containing primary afferent fibres in the dorsal horn also indicates a central role for SS, possibly in nociception (see below).

1.2.1.3 Calcitonin gene related peptide

CGRP has been localised using immunohistochemistry to a large proportion of *drg* neurons of all sizes, and in a dense fibre network in the dorsal laminae of the spinal cord and in nerve fibres of peripheral tissues (Rosenfeld et al., 1983; Gibson et al., 1984b; Wiesenfeld-Hallin et al., 1984; Gibbins et al., 1985, 1987; Skofitsch & Jacobowitz 1985; Uddman et al., 1985; Lee et al., 1985; Lundberg et al., 1985; Wanaka et al., 1986; Kruger et al., 1988). mRNAs encoding both α and β CGRP are found in perikarya of *drg* and are present in overlapping but distinct cell populations (Noguchi et al., 1990; Mulderry, 1988; Gibson et al., 1988). In peripheral tissues, CGRP has been localised in axons of sensory neurons (Lundberg et al., 1985), around blood vessels (Lundberg et al., 1985; Hanko et al., 1985; Wanaka et al., 1985; Uddman et al., 1985; Wharton et al., 1986; Gibbins et al., 1985) and in humans, in the skin as free nerve endings around blood vessels, sweat glands, hair follicles and in a few Meissner's corpuscles (see Dalsgaard 1988; Gibbins et al., 1987a; Dalsgaard et al., 1988) although not all of this CGRP is of sensory origin (Landis & Fredieu, 1986; Lindh et al., 1987). Molander et al. (1987) has shown that CGRP-containing sensory neurons project to cutaneous, muscular and visceral nerves. Thus, the presence of sensory fibres containing CGRP in peripheral tissues and of a dense innervation of CGRP in the dorsal horn suggests a possible role for CGRP as a sensory neurotransmitter.

1.2.1.4 Cholecystokinin

Initial studies using antisera raised against CCK suggested that this peptide accumulated in ligated peripheral nerves (Lundberg et al., 1978)

and CCK was demonstrated in nerve fibres and *drg* (Larsson & Rehfeld, 1979). However, whilst the octapeptide (CCK₈) is the dominant CCK-like peptide in the CNS, the levels of CCK₈ in spinal ganglia were shown by other methods to be extremely low or non-existent (Marley et al., 1982; Schultzberg et al., 1982). Furthermore, capsaicin depletion of *drg* CCK immunoreactivity was not paralleled by a reduction in CCK₈ content of *drg* (Schultzberg et al., 1982). It has now been shown using immunohistochemistry that CCK immunoreactivity in rat *drg* (Ju et al., 1986) is abolished by preadsorption with CGRP, suggesting that these CCK immunoreactive neurons in fact contain CGRP or CGRP-like peptide. This finding has been confirmed by Hokfelt (1988) using more specific antisera to CCK, which detected no CCK immunoreactivity in primary sensory neurons.

1.2.1.5 Vasoactive Intestinal Polypeptide

VIP was first described in primary sensory neurons of sacral *drg* and in the proximal stump of splanchnic and sciatic nerves (Lundberg et al. 1978). In the spinal cord, VIP-containing fibres are found in Lissauer's tract and in the most dorsal areas of lamina I; relatively higher levels of VIP are present in the lumbosacral region of the spinal cord (Lundberg et al., 1979; Annand et al., 1983, 1984; Basbaum & Glazer, 1983; Honda et al., 1983; Kawatani et al., 1983) localised to areas where visceral afferents terminate (Kawatani et al., 1983); but VIP has been identified in thoracic *drg* of colchicine treated cats (Kuo et al., 1985). VIP, presumed to be of sensory origin, is also present in peripheral tissues, in free nerve endings in the dermis-epidermis region and around hair follicles in the human skin (Björklund et al., 1986; Dalsgaard et al., 1988). In cat spinal cord, VIP is released into the dorsal horn upon electrical stimulation of the sciatic nerve at above C/A δ fibre threshold, and depolarises dorsal horn neurons to increase their excitability (Jeftinija et al., 1982).

1.2.1.6 Bombesin

Bombesin is a tetradecapeptide originally isolated from the skin of the frog *Bombina bombina* (Anastasi et al., 1971). Whilst antisera raised against bombesin recognise a mammalian form of this peptide in the spinal cord (Moody et al., 1981) and sensory neurons (Fuxe et al., 1983; Massari et al., 1983; Panula et al., 1983), the precise structure of mammalian bombesin is not clear. It has been suggested that the 27 residue gastrin-releasing peptide (GRP; McDonald et al., 1979) isolated from porcine gastric tissue and Neuromedin B (NMB) characterised from porcine spinal cord, which shows striking sequence homology with bombesin and elicit effects similar to that of bombesin (Minamino et al., 1983; Namba et al., 1984), are the mammalian equivalents. In rat spinal cord, bombesin-like immunoreactivity is found in nerve fibres, is in higher concentration in the dorsal horn (laminae I and II) than in the ventral horn and is released by veratridine and K^+ in a Ca^{2+} dependent process. In lumbosacral segments of spinal cord, bombesin immunoreactive fibres are found in greater numbers than in cervical or thoracic segments (Moody et al., 1981; Namba et al., 1985). O'Donohue et al. (1984) has found bombesin binding sites localised to the outer laminae of the dorsal horn, which correlates with the distribution of bombesin-containing fibres. Finally, it should be noted that the tachykinins NKA and NKB share the two C terminal amino acids with the bombesin/GRP family and the possibility that the bombesin-like immunoreactivity is due to cross-reaction of the antisera cannot be discounted (see Dalsgaard, 1988). Fortunately, the isolation of cDNA clones for these peptides has allowed the unambiguous localisation of the mRNA for both peptides using *in situ* hybridisation. It is found that only the mRNA for NMB is present in a subset of *drg* cells (Wada et al., 1990).

1.2.1.7 Opioid Peptides

A number of opioid peptides have been identified in *drg* in a variety of species using immunohistochemistry and radioimmunoassay. Low levels of dynorphin₁₋₁₃ have been found in extracts of rabbit *drg* (Botticelli et al., 1981) but in the spinal cord levels of dynorphin₁₋₁₃ were unaltered following dorsal rhizotomy; dynorphin has also been detected in cultures of *drg* (Sweetnam et al., 1982). Prodynorphin has been localised to a population of small somatosensory primary afferents of the guinea pig (Weihe et al., 1985); cutaneous afferents and afferents projecting to the pelvic viscera and airways also contain dynorphin (Gibbins et al., 1987). Senba et al. (1982) has identified a small population of sensory neurons in *drg* containing leu-enkephalin, although as dynorphin contains the leu-enkephalin sequence at its N-terminus, it is not clear if the leu-enkephalin detected in this study was, in fact, dynorphin cross reactivity. β -endorphin has been identified in cultured human *drg* (Kim et al., 1984).

1.2.1.8 Other peptides

In L5 *drg* of the rat, both OXY and AVP have been localised using immunohistochemistry, and identified using reversed phase HPLC, but the functional role of these two peptides remains unclear (Kai-Kai et al., 1985, 1986). GAL is a 29 residue peptide (Tatemoto, 1983) which has been immunohistochemically localised to a subset of primary sensory neurons (Ch'ng et al., 1985; Skofitsch & Jacobowitz 1985a; Ju et al., 1987). Whilst the peripheral projection and function of GAL is unclear, in the substantia gelatinosa, terminals containing GAL and a basal release of GAL have been reported (Ch'ng et al., 1985; Morton & Hutchison, 1989). CRF containing cell bodies have been reported in *drg* and capsaicin-sensitive fibres are reported in the dorsal horn (Schipper et al., 1984; Skofitsch et al., 1984, 1985). However, it has been suggested that CRF immunoreactivity in

primary sensory neurons is, in fact, due to cross-reaction with SP, despite their structural dissimilarity (Berkenbosch et al., 1986). In support of this, in preliminary studies for this thesis, I failed to detect any CRF in extracts of *drg* using RIA (G. Smith, unpublished observations). GHRH-like immunoreactivity has been reported in approximately 1% of all *drg* neurons; however, no fibres containing GHRH are present in the spinal cord or periphery and the possible role for GHRH remains obscure (Jozsa et al., 1987). Recently, mRNA and immunoreactivity for endothelin 1 has been localised to small and large neurons of *drg*, but immunoreactive fibres were rarely found in the dorsal spinal cord (Giaid et al., 1989). Finally, fibres containing ANF have been found in lamina I and II of the spinal cord and in 5% of the intermediate and large neuronal cell bodies in the guinea pig (Nohr et al., 1989).

1.2.2 Non-peptide transmitter candidates

1.2.2.1 Excitatory amino acids

Both L-glutamate (Glu) and L-aspartate (Asp) have been proposed as neurotransmitter candidates of *drg* neurons, and both have powerful excitatory actions on spinal neurons (Curtis et al., 1959). However, the available data tends to suggest that Glu is the endogenous transmitter acting on the high density of binding sites in the superficial dorsal horn (Greenamyre et al., 1984). Indeed, monosynaptic excitation of spinal dorsal horn neurones *in vivo* by physiological and electrical stimulation of large myelinated primary afferents is antagonised by iontophoretic application of antagonists of kainate and quisqualate receptors but not by antagonists specific for the NMDA receptor (Gerber & Randic, 1989; Yoshimura & Jessell, 1990; Dickenson & Sullivan, 1990); although both these compounds have been shown to be neurotoxic on the spinal cord (Urca & Urca, 1990). In contrast, frequency-dependent potentiation of dorsal horn

neuronal responsiveness to repeated C-fibre stimulation ('wind-up'; Mendell, 1966) is abolished by NMDA receptor antagonists (Davies & Lodge, 1987; Dickenson & Sullivan, 1990) which are also antinociceptive (Dickenson & Aydar, 1991). These results do not, by themselves, imply that Glu is released and acts postsynaptically, as these effects may be due to presynaptic inhibition of release of another transmitter (see Salt & Hill, 1983). However, dorsal horn neurons maintained in dissociated cell culture respond to application of Glu, but not Asp or NMDA (the specific agonist) with a rapid depolarisation of similar time course to the excitatory post-synaptic potential (epsp) elicited by sensory neuron stimulation (Jahr & Jessel, 1985).

Biochemical studies also suggest Glu may be the endogenous transmitter. The ubiquity of Glu and Asp as components of cellular proteins and metabolism make the interpretation of biochemical data more complicated than for most other neurotransmitter candidates. However, there is an excess of Glu in dorsal roots over ventral roots, which is not found for other amino acids (Roberts et al., 1973). An uptake system for Glu (and other amino acids) can be demonstrated in the spinal cord (see Salt & Hill, 1983) and [^3H]D-aspartate, which is internalised only by neurons utilising excitatory amino acid transmitters but not metabolised, is retrogradely transported from the dorsal horn to exclusively label large perikarya of *drg* (Cuenod et al., 1982). Antibodies raised against enzymes of amino acid metabolism e.g., glutaminase (metabolising Glu) or aspartate aminotransferase (metabolising Asp) have also proved useful. Immunohistochemistry for these enzymes reveals that aspartate aminotransferase is evenly distributed in *drg* cells but that a population of small- to medium-sized *drg* cell bodies constituting approximately 30% of all cells are selectively labelled by antibodies against glutaminase (Cangro et al., 1985). More recently, Battaglia & Rustioni (1988) have used specific

antisera raised against Glu to localise Glu in approximately 15-30% of *drg* neurons. The transport inhibitor colchicine increased the proportion of cells containing Glu to 70% but did not alter immunoreactivity for glutaminase. Furthermore, it has been suggested that as the intensity of Glu immunoreactivity does not correlate with cytochrome oxidase activity (a marker for cell metabolic activity) Glu immunoreactivity is not concerned with cell metabolism. Immunoreactivity for Glu has also been localised to approximately 8.5% of unmyelinated and 2.5% of myelinated axons in lumbar dorsal roots of the rat (Westlund et al., 1989). In studies using antisera raised against Glu and Asp fixation products, immunoreactivity for both was localised to the first three lamina of the dorsal horn, in a variety of subcellular structures including vesicles and synaptic densities (Merighi et al., 1991). Thus, whilst the evidence presented here does not rule out some other transmitter substance such as Asp, or the acidic dipeptide N-acetyl-aspartyl-glutamate (Westbrook et al., 1986) as the transmitter mediating the monosynaptic epsp, there is considerable support for Glu as the transmitter.

1.2.2.2 Purines

Electrophysiological studies indicate that ATP selectively excites a subpopulation of cultured dorsal horn neurons (Jahr & Jessel, 1983) and is found to increase the firing rate of neurons receiving primary afferent input in cuneate and caudal trigeminal nuclei (Galindo et al., 1967; Phillis & Wu, 1981). Earlier, it was found that antidromic stimulation of sensory nerves induced release of ATP at sites of termination in the periphery (Holton, 1959). Unfortunately, the ubiquity of ATP as the energy currency of the cell militates against immunohistochemical localisation of this candidate transmitter to *drg* neuron subpopulations.

1.2.3 Cytoplasmic markers for *drg* subpopulations

1.2.3.1 Fluoride resistant acid phosphatase (FRAP)

FRAP enzyme activity has been localised in a large subpopulation of small diameter primary afferent neurons in the rat (Kniyhar, 1971). In the spinal cord, FRAP activity is localised to lamina I and II and is completely abolished by capsaicin pre-treatment (Kniyhar-Csillick & Csillick, 1981; Nagy & Hunt, 1982; McDougal et al., 1985). FRAP activity has also been demonstrated in peripheral tissues (Kniyhar-Csillick & Csillick, 1981) and *drg* cells projecting in muscular, cutaneous and visceral nerves contain FRAP (Dalsgaard et al., 1984; Molander et al., 1987). The function of this non-lysosomal hydrolytic enzyme is unclear, but a number of substrates including thiamine monophosphate and guanine 5'-monophosphate are known; ATP, however, is not a substrate for FRAP (see Nagy & Daddona, 1985). FRAP activity labels approximately 75% of neurons with a dense cytoplasmic reaction product, whereas the remaining 25% exhibit staining of granules scattered throughout the cytoplasm.

1.2.3.2 Carbonic Anhydrase (CA)

CA catalyses the interconversion of CO_2 (gas) and H_2CO_3 and as such, is thought to be a marker of metabolically active cells. Cytochemical staining for CA in *drg* of a number of species is limited to larger diameter cells; smaller diameter cells containing little or no CA activity (Riley et al., 1984). In support of CA activity as a marker for metabolically active cells, the intensity of cytochrome oxidase staining (another measure of cell metabolic activity) is found to be correlated with CA staining (Carr et al., 1989).

1.2.3.3 Adenosine Deaminase (ADA)

ADA converts adenosine to inosine, and ADA activity is localised to a subpopulation of primary afferent neurons (Nagy et al., 1984). Such neurons are distinct from FRAP and 5' Nucleotidase (see below) populations, and terminals containing ADA activity have been localised to laminae I and II; capsaicin pre-treatment depletes ADA activity (Nagy & Daddona, 1985).

1.2.3.4 5' Nucleotidase (5'N)

5'N is involved in the catabolism of nucleotides and is localised in a distinct subpopulation of neurons in the *drg*; some 80% of cells containing 5'N activity also show FRAP activity (see Nagy & Daddona, 1985).

1.2.3.5 Cytoskeleton

The large light and small dark cell populations of *drg* can be distinguished using monoclonal antibodies raised against the 200kD neurofilament protein (RT97) which selectively label the large light neurons (Lawson et al., 1984). Another antibody, MAb ICII, which recognises a filamentous antigen selectively labels small and intermediate-diameter *drg* neurons and shows little or no overlap with those cells labelled by RT97 (Dodd & Jessel, 1986).

1.2.4 Cell Surface Markers

A number of cell surface antigens have now been shown to be expressed in subsets of *drg*. The majority of the surface antigens are oligosaccharides; globoseries carbohydrate sequences, recognised by monoclonal antibodies such as stage-specific embryonic antigen (SSEA)-3 and SSEA-4, are expressed selectively in the cytoplasm and on the surface of about 10-15% of adult *drg* neurons, of mainly intermediate and large size

(Dodd et al., 1984). The central terminals for these neurons are localised to laminae I, III and IV of the dorsal horn, suggesting they are indicative of myelinated afferents of either high threshold mechanoreceptors or low-threshold cutaneous receptors (Dodd & Jessell, 1985; Tajti et al., 1988).

Monoclonal antibodies directed against lactoseries carbohydrates (A5, LD2, KH10, 2C5, LA4, FC10.2, Anti-Le) label a variety of subpopulations and proportion of mainly small *drg* neurons (Dodd & Jessell, 1985; Mollicone et al., 1986). Terminals expressing lactoseries carbohydrates are restricted to the superficial laminae of the dorsal horn, and in the periphery, terminals of primary sensory neurons have been found to express these same lactoseries carbohydrates (Dodd & Jessell, 1985). Such cell surface markers appear early in the development of sensory projections to the spinal cord, are conserved between species, and it may be that these oligosaccharide epitopes are of importance in establishing the proper connections between sensory neurons and receptive neurons in the *drg* (Regan et al., 1986; Mollicone et al., 1986; Dodd & Jessell, 1986).

1.2.5 Peptide Co-existence

That neuropeptides co-exist in *drg* neurons is suggested from studies in which the numbers of cells immunoreactive to each of a selection of neuropeptides has been shown to exceed the total number of cells present. However, whilst positive evidence regarding co-existence of neuropeptides may, on first consideration, seem to be incontrovertible, problems regarding cross-reactivity must be considered when interpreting co-localisation studies. Fortunately, consistent results, in a variety of species, now suggest that widespread co-localisation of neuropeptides does occur, and that this is not restricted to simply paired co-localisation, but cells containing a number of peptides have been described; even co-localisation in the same secretory granule has been reported (Merighi et al., 1988).

Initial studies of co-localisation of SP and SS in rat *drg* indicated that SP immunoreactivity was found in a population of small or intermediate sized cells (10-20% of all neurons) distinct from those containing SS (10% of all neurons; Hökfelt et al., 1976). Tuschler & Seybold (1985) confirmed the distribution of SP and the discrete populations of SP and SS containing cell bodies, but found SS in small diameter neurons only. More recently in the rat, Ju and colleagues (1987) have reported colocalisation of SP and SS in 5-10% of SS-containing cells and colocalisation of SP and GAL; no co-existence of GAL and SS was reported. Co-localisation of SP and SS and the absence of colocalisation of SS and GAL has also been reported for cat (Garry et al., 1989) and Merighi et al. (1988) have reported colocalisation of SP and SS in the same secretory granule. In cat, SS is also colocalised with SP, VIP and 'CCK' (probably CGRP); and some cells may contain all four peptide immunoreactivities (Leah et al., 1985). *In situ* hybridisation for β -PPT mRNA and preproSS mRNA in rat *drg* showed that peptide immunoreactivity was found in the same cells expressing mRNA for the respective peptide; although no data regarding colocalisation of mRNA was reported in this study (Henken et al., 1988). Colocalisation of SS with CGRP in rat, cat, horse and pig has also been reported to the extent that the majority of SS immunoreactive cell bodies were also immunoreactive for CGRP (Ju et al., 1987; Merighi et al., 1988; Merighi et al., 1990; Garry et al., 1989). In the cat, colocalisation of SS with Leu-enkephalin has also been reported (Garry et al., 1989).

SP has been reportedly colocalised with a large number of neuropeptides. In the rat and cat, colocalisation with SS has been discussed above; but in the rat, SP immunoreactivity was found in cells containing CGRP and GAL (in fact, most SP cells contained CGRP; Ju et al., 1987; Skofitsch & Jacobowitz, 1985b). In the cat, SP was found colocalised with CGRP and Leu-enkephalin (Garry et al., 1989), and in

lumbar and sacral *drg*, it has been reported that SP immunoreactive cells can contain up to three other peptides; 'CCK' (probably CGRP), SS and VIP (Leah et al., 1985). SP immunoreactivity in horse and pig has also been colocalised with CGRP, GAL, enkephalin and combinations of SP, CGRP and enkephalin and SP, CGRP and GAL were also noted (Merighi et al., 1990). SP has also been colocalised with NKA (Dalsgaard et al., 1985), AVP and OXY (Kai-Kai et al., 1986), Glu (Battaglia & Rustioni, 1988), bombesin-like immunoreactivity (Dalsgaard et al., 1983; Fuxe et al., 1983) dynorphin (Gibbins et al., 1987) and endothelin (Giaid et al., 1989), but not ANF (Nohr et al., 1989). The mRNA for β -PPT has been shown to colocalise with SP-like immunoreactivity (Henken et al., 1988) and in all cells containing endothelin 1 mRNA transcripts (Giaid et al., 1989).

CGRP, the most abundant of neuropeptides in the *drg*, is contained in between 50% and 70% of *drg* neurons of diameter between 15 μ m and 65 μ m (Ju et al., 1987). Thus, it is not restricted to small dark neurons but is also present in large light neurons. It is, therefore, of little surprise that CGRP has been widely colocalised with a number of other neuropeptides in *drg*. Colocalisation with SP and SS in rat, cat and other species has been discussed above. In addition, in *drg* of rat, CGRP has been colocalised with GAL and VIP and in some cells, with both GAL and SP (Ju et al., 1987); in guinea pig colocalisation of CGRP with dynorphin and SP often in the same cell has been reported (Gibbins et al., 1987); colocalisation with VIP in the human has also been reported (Dalsgaard et al., 1988). Studies of the localisation of mRNA encoding α and β CGRP showed that whilst small and medium diameter neurons expressed both types of mRNA, larger neurons expressed a predominance of α CGRP mRNA; the colocalisation of both α and β CGRP mRNA was also noted (Mulder et al., 1988; Noguchi et al., 1990).

1.3 ROLE OF SP, SS AND CGRP IN THE *drg*

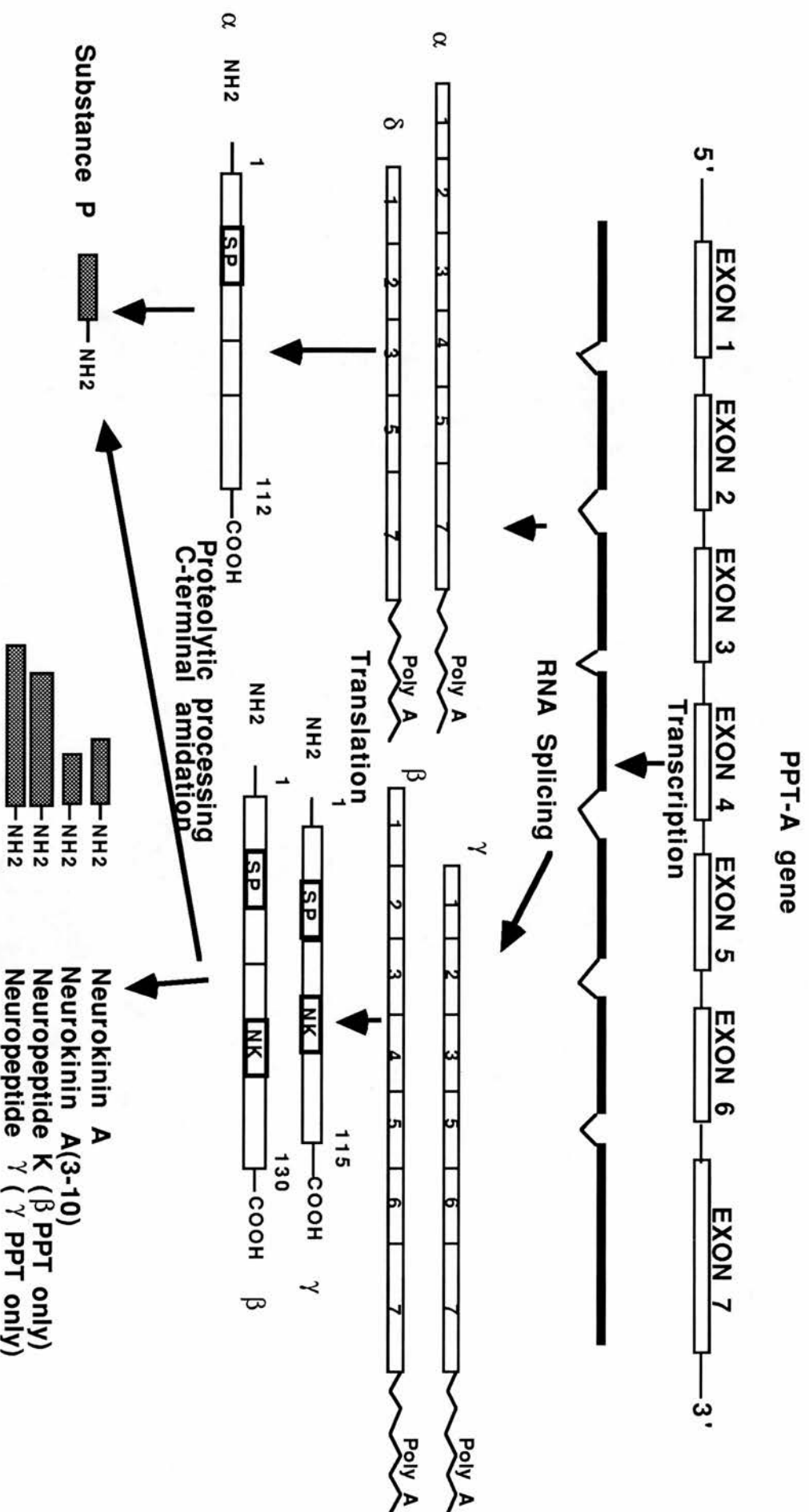
1.3.1 Substance P (SP)

Whilst SP was first isolated by von Euler & Gaddum in 1931 (see Nakanishi, 1987; Helke, 1990), it was only recently that the peptide was purified and its structure elucidated as the undecapeptide Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂ (Chang, 1971). SP is a member of the tachykinin family of peptides (literally 'fast-acting'), characterised by the common carboxy terminus Phe-X-Gly-Leu-Met-NH₂ where X is a hydrophobic or aromatic residue (Erspamer, 1971). Until recently, SP was the only known mammalian tachykinin, the others in the family having been isolated from octopod salivary glands or amphibian skin. However, a number of other tachykinins have now been demonstrated in the mammalian CNS (see Harmar, 1984; Maggio, 1988; Helke et al., 1990) and the existence of a number of preprotachykinin (PPT) mRNAs and two tachykinin genes has also been shown.

The PPT-A gene is approximately 8.4kbp long and consists of seven exons separated by six introns (Fig. 1.1); exons 2-7 encode six protein sequences as follows: signal peptide, SP, two spacer sequences, NKA (substance K) and the carboxy terminal sequence respectively (Nawa et al., 1983; Krause et al., 1987; Harmar et al., 1986). Alternative splicing of the primary transcript of the tachykinin A gene yields a number of mature mRNA species: α -PPT mRNA contains exons 2-5, but not 6, and exon 7, and thus encodes SP only, β -PPT mRNA consists of all exons 2-7 and thus, encodes SP and NKA and γ -PPT mRNA which does not contain exon 4 also encodes SP and NKA (Nawa et al., 1984; Krause et al., 1987). Recently, a fourth PPT mRNA, δ -PPT in which both exons 4 and 6 are absent has been isolated and sequenced (Harmar et al., 1990). Translation of α , β and γ preprotachykinin mRNA gives rise to precursor peptides of 112, 130 and 115 amino acids, respectively. Post-translational processing of the α peptide

Figure 1.1 Schematic diagram of the transcription and splicing of the rat preprotachykinin A gene. The rat tachykinin A is depicted at the top with the boxes denoting exons; transcription products of the genes are also shown. The mRNAs are translated with concomitant signal peptide cleavage, and the various peptides derived from each precursor are displayed

Fig 1.1. Tachykinin gene structure, transcription, translation and proteolytic processing



yields only SP. The β and γ peptides can also yield SP, but in addition, NKA and NKA (3-10) can also be produced through post translational processing. β propeptide also gives rise to an NH₂-terminally extended form of NKA (known as neuropeptide K) and the γ peptide yields another NH₂-terminally extended form (neuropeptide γ ; see Helke et al., 1990). It has been predicted that in addition to SP, δ -PPT may encode a novel C-flanking peptide of 22 amino acids (Harmar et al., 1990).

NKB (neuromedin K), a further mammalian tachykinin, is encoded by both of the two known transcripts of the PPT-B gene (Kotani et al., 1986). In common with the PPT-A gene, the PPT-B gene consists of 7 exons interspaced by 6 introns and gives rise to two PPT-B mRNAs, through use of different promoter sites, both containing exons 1-7 (NKB is contained in exon 5). The minor species of mRNA differs from the major in having two extra 5' exon sequences and lacking the 5' terminus of the first exon sequence of the major transcript. Translation of both mRNA species yields a propeptide of 126 amino acids containing the NKB sequence. NKB is thought to be the sole biologically active peptide product of the tachykinin B gene (Kotani et al., 1986).

Cell bodies and nerve terminals containing immunoreactive SP have been demonstrated in many regions of the CNS, most notably laminae I and II of the dorsal horn of the spinal cord, the caudate nucleus, the substantia nigra, the habenulointerpeduncular tract and intermediolateral cell column. In the peripheral nervous system autonomic ganglia, sensory ganglia (see above) and enteric neurons have also been found to contain immunoreactive SP (Brownstein et al., 1976; Ljunghdahl et al., 1978; Nicoll et al., 1980). Such studies employed radioimmunoassay of microdissected tissue or immunohistochemistry of brain sections. However, as SP is a member of a family of tachykinins, some only identified latterly, and each with a homologous carboxy terminal region, antisera raised

against one may (and often do) cross react with other peptides in the family. Thus, immunochemical data must be interpreted with caution as demonstrating the discrete distribution of one tachykinin peptide. Combining radioimmunoassay with chromatographic techniques has explicitly shown SP, NKA and neuropeptide K in spinal cord and *drg* (Ogawa et al., 1985; Arai et al., 1986; Too et al., 1989). Antisera to SP, shown not to cross-react with NKA and *vice versa* reveal a similar distribution of SP and NKA immunoreactivity in the spinal cord (see Helke et al., 1990), although the other mammalian tachykinins were not accounted for in this study.

In situ hybridisation for PPT-A and PPT-B mRNA can give some indication as to the likely distribution of NKB vs. NKA and SP. In the rat CNS, distinct patterns of expression of the two genes are found. Thus, for example, in spinal cord laminae I and II, PPT-A gene expression is prominent, whereas the PPT-B gene is predominately expressed in lamina III only. In the caudate nucleus numerous cell bodies containing PPT-A mRNA are found, whereas PPT-B expressing cells are located in the striatum (Warden et al., 1988). Interestingly, in the medial habenula, mRNA of both genes are co-localised (Burgunder et al., 1989).

The distribution of the three mRNA encoded by the PPT-A gene, α , β and γ has also been examined, by Northern blot and *in situ* hybridisation. In bovine tissues, tissue-specific distribution of PPT-A mRNAs has been reported, with β -PPT predominating in nervous tissue and α -PPT predominating in thyroid and gut (Nawa et al., 1984). In rat tissues, γ -PPT is the most abundant form and the splicing pattern of the PPT-A gene is thought to be relatively constant in all tissues studied (see Harmar et al., 1990). To date, δ -PPT mRNA has only been reported in rat *drg* (Harmar et al., 1990).

Mammalian tachykinins act through three tachykinin receptors originally distinguished using the amphibian tachykinins, physalaemin, eledoisin and kassinin in smooth muscle preparations and in ligand binding studies (see Buck & Burcher, 1986; Mizrahi et al., 1985). More recently, cDNAs encoding receptors binding SP (NK1), NKA (NK2) and NKB (NK3) have been isolated and sequenced from rat cDNA libraries (Yokota et al., 1989; Masu et al., 1987; Shigemoto et al., 1990). The three receptors show sequence similarities with some divergence, but all conform to the so called 7 transmembrane arrangement and belong to the family of G protein-coupled receptors. More recently, a putative G-protein-coupled receptor was isolated from a rat forebrain cDNA library using a probe for a particularly well conserved sequence within the transmembrane region, TM6, of the G protein coupled receptors. Sequence comparison showed that this clone encoded a polypeptide containing seven transmembrane domains, showing greatest homology with tachykinin receptor sequences (Eva et al., 1990). It is possible, therefore, that a fourth tachykinin receptor may exist.

The distribution of tachykinin binding sites has been investigated using Northern blot hybridisation of probes specific for each of the three receptors, and autoradiography of a number of radiolabelled ligands with some specificity for each of the three known subtypes. The distribution of binding sites in the spinal cord is discussed below. In the CNS, NK-1 binding sites have been localised to neurons and glia, and are present in high density in the caudate putamen, but in relatively low abundance in the substantia nigra (Mantyh et al., 1989; Buck et al., 1986). NK-1 binding sites are also present on neurons of the myenteric plexus, smooth muscle cells, fibroblasts and a number of circulating inflammatory and immune cells (Mantyh et al., 1989). The NK-2 receptor is present in highest concentration in peripheral tissues, in a pattern distinct from that of NK-1 receptors, and interestingly associated with smooth muscle of blood vessels (Mantyh et al.,

1989). In the CNS, NK-2 binding sites are reported in highest density in the external plexiform layer of the olfactory bulb, with lower densities in the caudate nucleus, substantia nigra and the striatum (Dam et al., 1990). However, some of these sites may have been NK-3, and some authors suggest NK-2 binding sites may be exclusively in peripheral tissues (Mantyh et al., 1989; Saffroy et al., 1988). The NK-3 receptor is found primarily in the CNS, although neurons of the rat myenteric plexus and smooth muscle cells of the rat portal vein reportedly contain NK-3 binding sites (Mantyh et al., 1989; Buck et al., 1986; Saffroy et al., 1988). In the CNS, NK-3 binding sites are concentrated in the deep cortical layers (Mantyh et al., 1989), and in the nigrostriatal system, the distribution of NK-3 binding sites reflects that of NK-2 sites (see above).

1.3.1.1 Central role of SP

Evidence supporting a role of SP in the *drg* as a sensory transmitter was obtained from early studies indicating that dorsal roots contained greater SP content than the ventral roots (Lembeck, 1953) and that SP was released into the perfused frog spinal cord following electrical stimulation of the hindlimb (Angelucci, 1956). Release of SP from sensory terminals has now been demonstrated in the rat *in vivo* (Yaksh et al., 1980) and *in vitro* (Otsuka & Konishi, 1976; Theriault et al., 1979) from peripheral sensory fibres with conduction velocities in the A δ and C-fibre range (Yaksh et al., 1980). Intracellular recording from dorsal horn neurons in the rat *in vitro* has shown that the slow, synaptically-mediated depolarisation elicited by high-frequency dorsal root stimulation is mimicked by application of SP (Urban & Randic, 1984). Moreover, SP antagonists block this slow depolarisation elicited either by SP or dorsal root stimulation. Further support for SP as a sensory transmitter in the spinal cord comes from the distribution of tachykinin binding sites. NK₁ NK₂ and NK₃ type binding sites

have been identified in the spinal cord. The density of NK₁ binding sites in the spinal cord is greatest in laminae I and II, the intermediolateral cell column and lamina X. NK₂ binding sites are associated with the dorsal horn only, in lamina I-III and NK₃ binding sites are distributed similarly (Helke et al., 1986; Charlton & Helke, 1985, 1985a; Buck et al., 1986).

Iontophoretic application of SP onto the dorsal horn exerts a powerful excitatory action on a subpopulation of dorsal horn neurons which are also excited by a peripheral noxious stimulus (Henry, 1976; Randic & Miletic, 1977). Dorsal horn neurons responding to low threshold afferent inputs also respond to SP (Henry, 1982) and Leah and collaborators (1985a) found SP in afferents conveying both noxious and innocuous stimuli. More recently, SP was found to be released following noxious mechanical or noxious thermal stimulation (Kuraishi et al., 1985; Wiesenfeld-Hallin, 1986a) and noxious stimulation has also been found to lead to induction of the PPT-A gene in rat *drg* (Noguchi et al., 1988). The distinct localisation of SP immunoreactive fibres in laminae I and II (Henry, 1976), the area of termination of fine myelinated (A δ) and non-myelinated (C) fibres also supports a role for SP in the transmission of nociceptive information (Christensen & Perl, 1970). Behavioural studies have to be interpreted with caution; however, lumbar intrathecal injection of SP in conscious rats elicits caudally directed scratching, licking and biting behaviour consistent with perception of a noxious stimulus (Piercy et al., 1981; Hylden & Wilcox, 1981; Seybold et al., 1982; Wiesenfeld-Hallin et al., 1984). From the evidence above, it appears that SP containing primary afferents, terminating in the dorsal horn, may be involved in the transmission of noxious stimuli.

1.3.1.2 Peripheral role of SP

An important role for SP in peripheral tissues is implied from the finding that the majority of SP synthesised in the *drg* is transported in

peripheral compared with central nerve (Brimijoin et al., 1980; Harmar & Keen, 1982). In addition, a number of nerves of the periphery are known to contain SP, including the greater and lumbar splanchnic, phrenic and sciatic (see Dalsgaard, 1988). SP immunoreactive fibres are also found in most peripheral tissues; in the skin associated with blood vessels, sweat glands and hair follicles (Dalsgaard et al., 1983a). SP immunoreactive fibres are also present in dental pulp as free nerve endings and around blood vessels (Olgarth et al., 1977, 1977a; Brodin et al., 1981); in the rat mammary nipple (where it is associated with lactation; Traurig et al., 1984) and the cornea, uvea, iris and retina of the eye (see Dalsgaard, 1988). In the cornea, capsaicin pretreatment depletes 80% of the SP (Keen et al., 1982) and in the iris, trigeminal nerve denervation completely removes SP innervation (Butler et al., 1980; Tervo et al., 1982) suggesting in these tissues that SP is, in part at least, of sensory origin.

Blood vessels are richly innervated by dense networks of SP immunoreactive nerve fibres (Furness et al., 1982; Papka et al., 1984) which are depleted following capsaicin pretreatment (Furness et al., 1982). SP-containing fibres are also found in the nasal mucosa, respiratory tract, epithelium of the lung and tongue, taste buds, gastrointestinal tract, parotid gland, myocardium, endocardium, epicardium, bladder, kidneys, adrenal gland and pia mater of the brain and spinal cord. However, it should be noted that not all SP immunoreactive nerves are of sensory neuron origin (see Dalsgaard, 1988).

In 1901, Bayliss noted that a variety of mechanical, chemical and physical stimuli to the skin produced vasodilatation, and that this phenomenon was dependent on the integrity of sensory neurons. Hinsey & Gasser (1930) have shown that the nerve fibres mediating this increased blood flow are the C fibres of sensory neurons; neurogenic inflammation is prevented by capsaicin treatment or denervation (Jancso et al., 1967), which

also reduces SP in primary afferent neurons (Gamse et al., 1980). A number of lines of evidence support a role for SP in vasodilatation (see Pernow, 1985); the presence of SP in peripheral nerve terminals has been discussed above. Release of SP from peripheral branches of primary sensory neurons occurs in the cat tooth pulp following antidromic stimulation of the inferior alveolar nerve (Olgarth et al., 1977), and results in a depletion of 60% of the SP in this tissue. Similarly, in the skin, experimental blister fluid is found to contain SP following antidromic stimulation of the sciatic nerve (White & Helme, 1985). Antidromic stimulation of nerves innervating skin also elicits a plasma extravasation and vasodilatation (Hallberg & Pernow, 1975; Hagermark et al., 1978; Ferrell & Russell, 1985), similar to that seen following close arterial infusion of SP (Lembeck & Holzer, 1979). Both electrically and SP-induced hyperaemia is blocked by the SP antagonist (D-Pro², D-Trp^{7,8}) SP (Rosell et al., 1981) and pretreatment with capsaicin, which almost completely depletes SP in the skin, abolishes antidromic vasodilatation and plasma extravasation (Lembeck & Holzer, 1979; Gamse et al., 1980).

Further evidence for SP as a mediator of plasma extravasation comes from studies in the eye. Stimulation of the trigeminal nerve distal to the gasserian ganglion causes depletion of SP immunoreactivity and increases the SP content of venous effluent blood (Bill et al., 1979; Brodin et al., 1981). Topical application of mustard oil or other irritants also releases SP; and local administration of SP to the eye causes miosis, hyperaemia and damages the blood aqueous barrier (Mandahl & Bill, 1981). This is a direct action of SP as the responses are not significantly attenuated by nerve conduction blockade by tetrodotoxin or trigeminal denervation. The effects of noxious agents on the eye are attenuated by capsaicin pretreatment or SP antagonists (Bynke, 1983). Thus, evidence from studies in skin and the

eye suggests SP may be involved in the process of neurogenic inflammation.

The mechanism by which SP induces vasodilatation and hyperaemia is unclear, however SP is found to induce degranulation of mast cells with liberation of histamine and the leukotrienes LTB₄, LTC₄ and LTD₄ (Erjavec et al., 1981; Payan et al., 1984). Both histamine and LTB₄ stimulate release of SP from peripheral sensory nerve endings, and SP antagonists inhibit oedema formation by compound 48/80, a histamine liberator (Saria et al., 1984). Furthermore, histamine, LTC₄ and LTD₄ increase vascular permeability and LTB₄ stimulates influx and accumulation of endothelium adherent polymorphonuclear leukocytes and monocytes. However, SP may also have a direct action, as pretreatment with histamine receptor antagonists or depletion of histamine with compound 48/80 reduces but does not completely block plasma extravasation induced by SP or antidromic nerve stimulation (Lembeck & Holzer, 1979; Couture & Cuello, 1984). SP also induces chemokinesis of polymorphonuclear leukocytes and chemotaxis of monocytes and neutrophils (Wiedermann et al., 1989; Helme et al., 1987).

1.3.2 Somatostatin

SS was first isolated and sequenced as a tetradecapeptide from extracts of ovine hypothalami (Brazeau et al., 1973), and subsequently identified in a variety of other tissues (see Reichlin, 1986). In addition to the tetradecapeptide (SS-14), an amino-terminally extended form (SS-28) and a variety of prohormonal forms and other fragments have been identified (Esch et al., 1980; Pradyrol, 1980). The gene encoding SS has been isolated from a variety of species including anglerfish, catfish, and human (see Andrews & Dixon, 1986); fish express two SS genes, which give rise to distinct prepro SS I and II mRNA, encoding SS-14 and SS-28 respectively

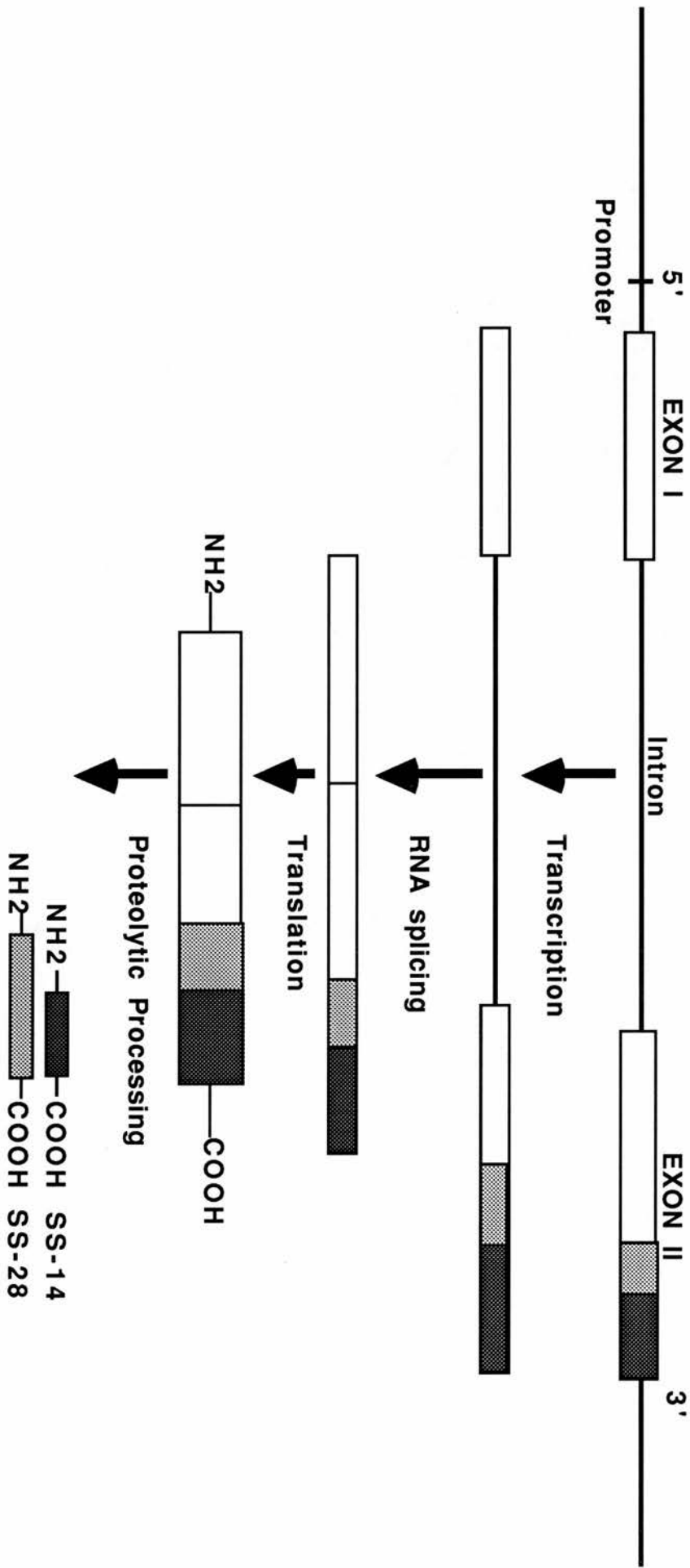
(Noe & Speiss, 1983). Mammalian tissues contain a single gene, yielding a prepro SS encoding both SS-14 and SS-28 (Shen et al., 1982; Tavianini et al., 1984).

The rat and human SS genes are similar and consist of two exons separated by a 621bp intron (see Fig. 1.2). The first exon contains a signal peptide and the sequences for SS-14 and SS-28 are found in exon 2. A prepro SS consisting of exon 1 and exon 2 results from transcription of the SS gene and, in turn, yields a 116 amino acid proSS, containing the SS-14 and SS-28 sequences at the carboxy terminus. The SS-14 sequence is preceded by two adjacent basic residues, whilst that of SS-28 is preceded by a single arginine. It is thought that proteolytic cleavage occurs to release either SS-28 or SS-14, although the enzymes involved and factors regulating this process are not known. Tissue-specific processing to either SS-14 or SS-28 has been shown in a number of tissues including *drg* and side-chain modification occurs in fish (see Andrews & Dixon, 1986).

Since the identification of SS, the peptide has been ascribed a wide variety of functions in both peripheral and central systems (see Reichlin, 1986). SS containing fibres form part of the complex that terminates in the median eminence, and inhibits growth hormone secretion. Hypothalamic SS is also one of a number of factors that regulate thyroid stimulating hormone secretion. In the pancreatic islets, SS contained in D cells inhibits release of insulin (B cells) and glucagon (A cells) in what is thought to be a paracrine role, although a genuine endocrine role cannot be excluded. SS also has a large number of effects on the gut, inhibiting exocrine secretions and release of a number of gut peptides, including gastrin, CCK, VIP, glucagon and secretin. In addition, it may also inhibit duodenal motility, gall bladder contraction and gastric emptying (see Bethge et al., 1982). In the nervous system outwith the hypothalamus, SS has been found in significant concentration in the cortex, septum, preoptic area and the thalamus. Lower

Figure 1.2. Schematic illustration of the transcription and splicing of the rat SS gene primary transcript. The gene is depicted at the top with the numbered boxes, denoting exons. The single transcription product is shown, this is translated with concomitant signal peptide cleavage, and the possible post-translational processing products, SS-14 and SS-28 are also shown

Fig 1.2. The Rat Somatostatin Gene



concentrations of SS are found in midbrain and brainstem nuclei, striatum, cerebellum and olfactory bulb (Brownstein et al., 1975).

Somatostatin is thought to produce its biological effects by binding to high affinity membrane receptors coupled to one or more pertussis toxin-sensitive G proteins. It is not clear if different SS receptors exist for the various forms of SS found in mammalian tissues; however, in rat anterior pituitary, a number of proteins have been specifically labelled using radiolabelled SS ligands and affinity cross linking methods (see Brown et al., 1990). More recently, photoaffinity labelling studies have identified an 85kDa protein in GH₄C₁ pituitary cell line membranes as a SS receptor. In a number of other cell lines, proteins of slightly different molecular weight were labelled, although this was thought due to differences in glycosylation (Brown et al., 1990).

1.3.2.1 Role of SS in the *drg*

In the spinal dorsal horn, SS-containing fibres are present in laminae I and II (Hökfelt et al., 1975, 1976; Dalsgaard et al., 1981; Nagy et al., 1981); some, but not all (Ribeiro-da-Silva & Cuello, 1990), are of primary afferent origin (see above), and it has been suggested SS may be involved in sensory transmission. Using antibody microprobes, Morton et al. (1989) have shown that the basal release of SS in the region of the substantia gelatinosa is increased following noxious thermal stimulation, but not innocuous thermal or mechanical stimuli or noxious mechanical stimuli. Similarly, release of SS into rabbit dorsal horn was increased by noxious thermal, but not mechanical, stimuli (Kuraishi et al., 1985). Furthermore, SS binding sites have been localised to the spinal cord of rat and human using radiolabelled SS-14 and SS-28. Binding site density is particularly pronounced in the substantia gelatinosa and lamina X, supporting the

suggestion that SS may be involved in sensory transmission (Reubi & Maurer, 1985; Reubi et al., 1986; Leroux et al., 1985).

Intrathecal administration of SS into the lower lumbar spinal cord produces a caudally directed behaviour consistent with perception of irritation and possibly pain, and increases the excitability of a flexion reflex to noxious thermal but not to mechanical stimuli (Seybold et al., 1982; Wiesenfeld-Hallin, 1985; Cridland & Henry, 1988; Wiesenfeld-Hallin, 1986). Intrathecal administration of anti-SS antiserum significantly inhibits the nociceptive response to thermal, but not mechanical stimulation (Ohno et al., 1988). SS also influences the firing of dorsal horn neurons (Randic & Miletic, 1978) and SS applied topically to the spinal cord is analgesic in animals (Mollenholt et al., 1988) and human (Meynadier et al., 1985; Chrubasik et al., 1984); this variation in the results of intrathecal administration of SS may be due to the variety of doses used. However, SS also causes hind limbs paralysis and neuronal damage, possibly through an ischaemic neurotoxic action and thus analgesia may be due to a pathological, rather than physiological, action of SS (Long, 1988; Mollenholt et al., 1988; Gaumann & Yaksh, 1988). More recently, it has been suggested that analgesia is elicited by non-neurotoxic intrathecal doses of SS (Mollenholt, 1990).

Intradermal injection of SS elicits a wheal and flare reaction in the skin of the human forearm, and the flare, but not the wheal, is dramatically reduced by capsaicin pretreatment, suggesting part of the action of SS is mediated by primary afferent C-fibres (Anand et al., 1983). Whilst SS containing fibres are found surrounding cutaneous blood vessels (O'Shaughnessy et al., 1983), SS is also found to release histamine from mast cells (Theoharides et al., 1981) and it may be that this mechanism underlies the action of SS in peripheral inflammation.

1.3.3 Calcitonin gene related peptide

CGRP is a 37 amino acid peptide encoded by the calcitonin gene and produced as a result of alternate post transcriptional splicing of the calcitonin gene primary transcript (see Zaidi et al., 1987). The peptide was discovered when a spontaneous and permanent switching from high to low calcitonin production in a rat medullary thyroid carcinoma cell line was found to be due to a switching of production from a calcitonin associated mRNA to a transcript approximately 200 nucleotides longer (Rosenfeld et al., 1981). Subsequent investigation revealed that this new transcript encoded a 128 amino acid precursor peptide (Rosenfeld et al., 1983) and was due to alternate post-translational processing of the primary transcript (Amara et al., 1982). More recently, Amara et al. (1985) and other groups have identified a second closely related sequence in rat and human and now two calcitonin/CGRP genes are known; the α and β respectively (Steenbergh et al., 1985; Alevizaki et al., 1986).

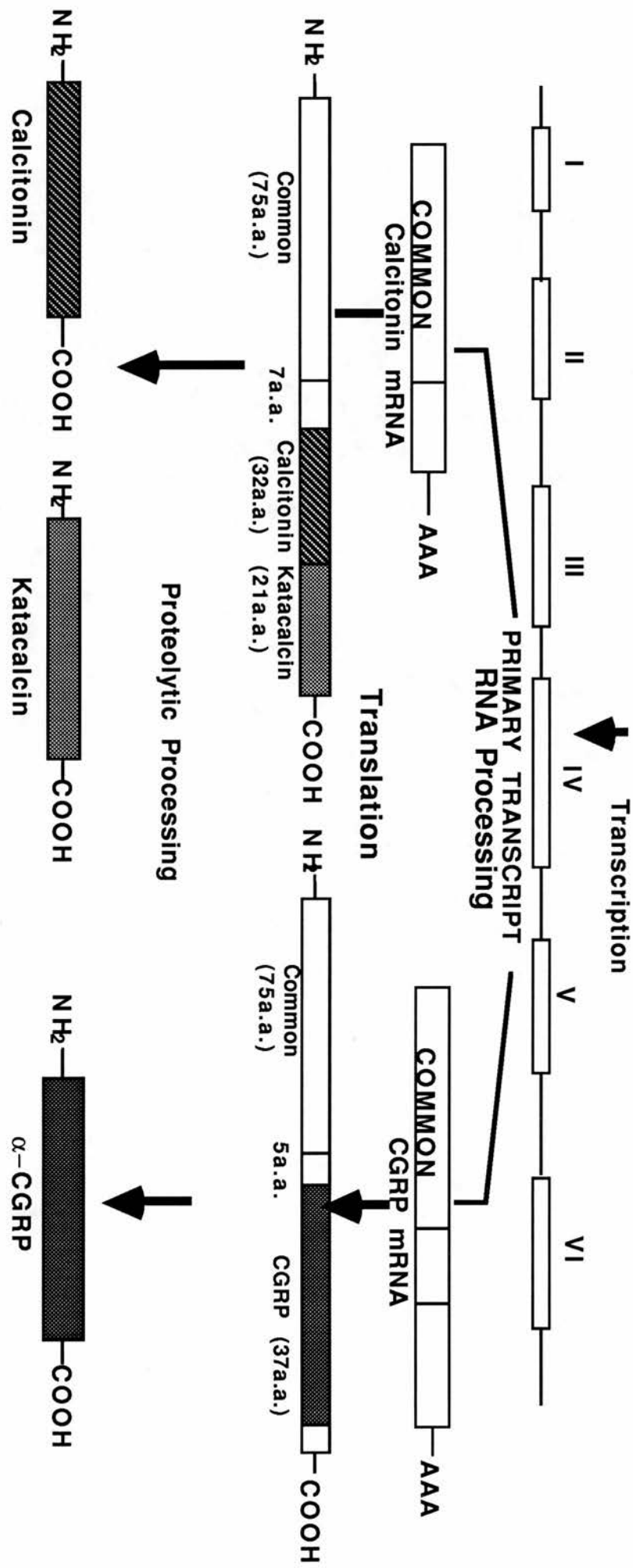
The α -calcitonin gene is found on the short arm of chromosome 11, consists of six exons and is about 6.5kbp long. The first three exons are common to both calcitonin and CGRP mRNA (although exon 1 is not translated), whilst the 4th exon contains the sequences for katacalcin and calcitonin, and an untranslated sequence at the end of which is a polyadenylation signal. The α CGRP mRNA consists of the first three exons, exon five containing the α CGRP sequence and exon six, which is not translated and has the alternative polyadenylation signal (see Fig. 1.3). Evidence that the different transcripts arise from alternative post-translational splicing and are not due to differentially directed transcription from the gene comes from the finding that nascent transcripts prepared from nuclei of tumours producing either calcitonin or CGRP mRNA show no difference (see MacIntyre et al., 1987).

Figure 1.3

Schematic illustration of the transcription and splicing patterns of the human α CGRP gene is depicted at the top with the numbered boxes, denoting exons; transcription products of the genes, arising through alternate splicing are also shown. The mRNAs are translated with concomitant signal peptide cleavage, and the peptide products are shown



Fig 1.3. The Human calcitonin/ α-CGRP gene



The β calcitonin/CGRP gene is also localised to the short arm of chromosome 11 and is thought to have arisen due to exon duplication. Whilst exons three and five share 92% homology, the non-coding regions (exons 1 and 6) are divergent. Exon four (containing the calcitonin sequence in the α calcitonin/CGRP gene) is only 65% homologous with the α and is unlikely to be translated as the reading frame terminates after only eight amino acids. Furthermore, the polyadenylation signal is found to be significantly altered in exon four of the β gene further reducing the likelihood of transcription. It may be that the β Calcitonin/CGRP gene is committed to CGRP production alone. The rat α and β CGRP sequences differ by only one, conservative, amino acid substitution (an Asp at position 25 in α is replaced by a Asn in β), suggesting that functionally they may subserve similar or identical functions (see Zaidi, 1987; MacIntyre et al., 1987).

Despite its relatively recent discovery, CGRP has been shown to exert a wide variety of biological activities including inhibition of gastric acid secretion, cardiac acceleration, vasodilatation, regulation of calcium metabolism, modulation of blood pressure, and body temperature and modulation of nociception (see Dennis et al., 1990). The peptide is widely distributed throughout the body, found in many of the areas of the CNS, in the spinal cord, thyroid gland, stomach, duodenum, pancreas and kidney (Skofitsh & Jacobowitz, 1985; Okimura et al., 1987; Wimalawansa et al., 1987). On the basis of the activity of a C-terminal fragment of CGRP, CGRP₈₋₃₇ in a number of *in vitro* bioassay systems at least two CGRP receptors are thought to exist (Dennis et al., 1990), although these are not thought to distinguish the α and β forms of CGRP (Zaidi et al., 1990; Foord & Craig, 1987).

1.3.3.1 Central role of CGRP

CGRP has been localised to *drg* and in a dense network of fibres in the dorsal laminae of the spinal cord (Rosenfeld et al., 1983; Gibson et al., 1984b; Wiesenfeld-Hallin et al., 1984; Gibbins et al., 1987; Skofitsch & Jacowbowitz, 1985b; Lee et al., 1985; Kruger et al., 1988). Furthermore, 'CCK'-like immunoreactivity (probably CGRP, see above) is found in both noxious and non-noxious primary afferents, and CGRP binding sites have been localised to the spinal dorsal horn, suggesting it may play some role in transmission of sensory information from the periphery to the spinal cord (Leah et al., 1985a; Henke et al., 1985). Immunoreactivity in the dorsal horn of the rat is increased following electrical stimulation of the sciatic nerve (Klein et al., 1990).

Administered intrathecally, CGRP does not of itself elicit any behavioural response, but the scratching, biting and licking behaviours observed following intrathecal administration of SP, NKA or SS are potentiated (Wiesenfeld-Hallin et al., 1984, 1986; Woolf and Wiesenfeld-Hallin, 1986); similarly, intraplantar injection of CGRP sensitises, but does not induce, hyperalgesia (Nakamura-Craig & Kaur-Gill, 1991). It is not clear how CGRP produces this effect, but it may be, as has been suggested, that it inhibits enzymatic breakdown of the other peptides (Le Greves et al., 1985; Le Greves et al., 1989). Alternatively, it might directly affect the dorsal horn neurons responding to these other peptides to lower their threshold to respond. Another possible mechanism of action of CGRP proposed by Oku et al. (1987), that CGRP potentiates release of SP in the dorsal horn, is evidenced from studies of capsaicin-induced release.

1.3.3.2 Peripheral role of CGRP

In the periphery, CGRP is found in terminals of *drg* (Dalsgaard et al., 1988; Lundberg et al., 1985; Wanaka et al., 1986; Uddman et al., 1985;

Gibbins et al., 1985, 1987) and both α and β forms have been implicated in the process of inflammation (Brain et al., 1985, 1986a,b; Gamse & Saria, 1985; Brain & Williams, 1985; Weihe et al., 1988). Following antidromic nerve stimulation, CGRP, a potent vasodilator (Brain et al., 1985) is released into the periphery (Saria et al., 1986) and whilst not inducing plasma extravasation itself, enhances oedema induced by SP and other mediators of increased microvascular permeability (Brain & Williams, 1985; Gamse & Saria, 1985). It has been suggested that CGRP vasodilatation is mediated by a release of relaxing factors as the vasodilatation appears endothelium-dependent (Brain et al., 1985). However, in the bovine coronary artery, vasodilatation in response to CGRP does not require the presence of an intact endothelium (Greenberg et al., 1987). Further support for a direct action of CGRP on endothelial-smooth muscle comes from the finding that a receptor for CGRP is found in such tissue (Hirata et al., 1988). Interestingly, in rat lung, CGRP inhibits biosynthesis of the inflammatory mediator LTD₄ (Di Marzo et al., 1986) and in guinea pig ileum, LTD₄-mediated contractions are inhibited by CGRP (Tippins et al., 1986).

1.4 ADRENAL STEROIDS

Of the 50 or so different steroids isolated from adrenal sources, only a small number are secreted into the venous blood and fewer still have ascribed biological activity, the remainder being biosynthetic intermediates, products of catabolism or with poorly understood activities. The two major steroid groups secreted by the adrenal gland are the glucocorticoids and mineralocorticoids synthesised in the *zona fasciculata*, and *zona reticularis* of the adrenal gland respectively. Whilst it is an oversimplification, glucocorticoids influence carbohydrate and protein metabolism and have anti-inflammatory activity, whereas mineralocorticoids are concerned with electrolyte balance, especially Na⁺ and K⁺. Other steroids produced by the

adrenal gland include androgens, progestogens and oestrogens. In rat, but not man, the major secreted corticosteroid is corticosterone, with large amounts of 18-hydroxy-11-deoxycorticosterone also present; 18-hydroxycorticosterone and aldosterone (a mineralocorticoid) are also secreted, but in smaller amounts (see Wilson & Foster, 1985; Leung & Munck, 1975).

1.4.1 Mechanism of action of steroid hormones

The first indication that steroid hormones may produce their effects by acting at the level of the genome came from the observation that ecdysone induced 'puffs' in the giant chromosome of insects. It has subsequently been shown that corticosteroids bind with a cytosolic receptor which moves to the nucleus where it binds with chromatin. Much effort has been devoted to the characterisation and cloning of steroid receptors, the identification and cloning of hormone regulated genes and the characterisation of sequences in the vicinity of such genes that confer hormone responsiveness (see Carson-Jurica et al., 1990).

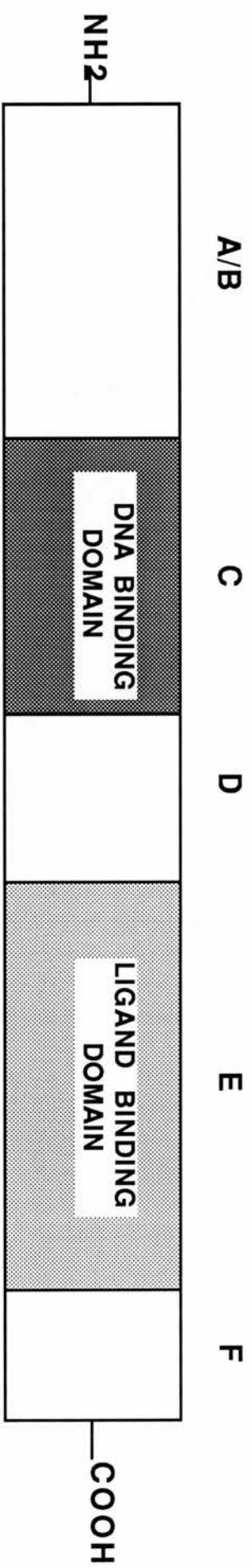
The mineralocorticoid (Type I) and glucocorticoid (Type II) receptors belong to a family of ligand-activated enhancer-binding factors (see O'Malley, 1990), which also includes receptors for the sex steroids, thyroid hormone, vitamin D₃ and retinoic acid, in addition to a number of oncogenes (e.g., v-erbA) and so-called orphan receptors, the ligands for which are unknown (e.g. COUPTF- the chicken ovalbumin upstream promoter-transcription factor). Cloning and sequencing of cDNAs for a number of the receptors in this family suggests they share a similar structure and may derive from a common ancestral origin.

The receptors are characterised as being composed of five structural domains designated A-E. Domains C and E are conserved between receptors; C, the DNA binding region, is particularly homologous, and

E forms the ligand binding domain. Domain D forms the hinge region between C and E; domains A/B located at the N-terminus, are hypervariable but their function is not fully understood. Closer analysis of domain C reveals nine conserved cysteine residues, eight of which form two zinc finger motifs, containing one zinc atom each. A series of experiments utilising domain swapping between receptor subtypes and site directed mutagenesis has shown that the first Zn finger is concerned with determining the sequence specificity of DNA binding and the second finger interacts less specifically and may stabilise dimer formation at steroid response elements. The ligand binding domain (E) contains a large number of hydrophobic amino acids and is particularly well conserved in two regions of 42 and 34 residues located in the middle of the domain. Whilst this high degree of conservation suggests that these two regions are functionally important, their role is not clear. The less well-conserved regions of this domain may confer ligand specificity on the molecule, but domain E is also important in ligand activated nuclear translocation. For the oestrogen receptor, which binds to DNA as a dimer (as does the GR), domain E is also important in the process of dimerisation (see Fig. 1.4; see Evans, 1988; Green & Chambon, 1988; Beato, 1989).

DNA sequences mediating steroid induction of gene transcription have been identified using gene transfer, site-directed mutagenesis and DNase protection techniques. This work has led to construction of a 15mer consensus sequence AGAACAnnnTGTTCT for the glucocorticoid response element (GRE). Induction by mineralocorticoids, androgen and progesterone is also possible through this sequence, although subtle differences in the detailed contacts each receptor makes with the GRE are found. That these differences are of functional importance, is suggested by the finding that mutation of individual sequences has differential effects on the response to different receptors. Response elements for other steroids

FIG 1.4. Domain structure of the Nuclear Hormone receptor superfamily



including oestrogen, thyroid hormone and retinoic acid show similar birotational dyad symmetry (see Beato, 1989).

In addition to upregulating gene expression, glucocorticoids can also repress gene transcription. Currently, only three examples of this process are known; the pro-opiomelanocortin gene, the prolactin gene and the gene for the α subunit of glycoprotein hormones. Analysis of the sequence recognised by the GR (GRE(-)) does not allow a clear consensus to be derived, however features in common with and divergent from the activating GRE can be identified. Whilst it may be that the binding of GR to GRE(-) may directly inhibit transcription (as is proposed for glucocorticoid repression of prolactin), such sequences are not common in glucocorticoid repressed genes. This has led to the suggestion that repression may be mediated by competition, between GR and other activators for binding sites on the 5' regulatory sequence of such genes. In the case of the α subunit gene, glucocorticoid inhibition is only apparent in constructs also containing cAMP response elements and in cells able to respond to cAMP. In other cell types, the same response elements can act as positive modulators of adjacent promoters, thus it may not be the nucleotide sequence *per se* that mediates repression, but rather its proximity to other regulatory elements that may be important (see Beato, 1989).

1.4.2 Anti-inflammatory activity of glucocorticoids

Glucocorticoids are potent anti-inflammatory agents used in the treatment of inflammatory disorders such as rheumatoid arthritis (McCarty, 1989). Despite their widespread clinical use, the mechanism of action of glucocorticoids is not clear. One suggestion promulgated over the previous decade or so, is that these steroids induce the biosynthesis of proteins which inhibit phospholipase A₂ (PLA₂) (see Flower, 1985, 1988). Previously, these proteins were referred to as macrocortin (Blackwell et al., 1980),

venocortin (Hirata 1981) or lipomodulin (Cloix et al., 1983), but are currently termed lipocortins (Flower, 1985). PLA₂ is presumed to initiate release of fatty acid substrates for synthesis of pro-inflammatory eicosanoids, and thus lipocortin, by inhibiting PLA₂, reduces the production of inflammatory mediators and the consequent inflammation.

Synthesis and release of lipocortin is greatly stimulated by glucocorticoids, such that following adrenalectomy (ADX), the concentration of lipocortin in the body fluids of animals is reduced (Blackwell et al., 1982). Furthermore, in a carrageenin-induced pleurisy model of inflammation in the rat, following ADX, the magnitude and duration of inflammation was approximately double, compared with that of sham operated animals (Flower et al., 1986). The concentration of pro-inflammatory eicosanoids such as 6-keto-prostaglandin F₁α, thromboxane B₂, and leukotriene B₄ was also enhanced (Flower et al., 1986). Glucocorticoid administration reversed the effects of ADX. More recently, the inflammatory reaction resulting, from subcutaneous injection of polyacrylamide suspension in the dorsal surface of mice, was strongly inhibited in a dose-dependent fashion by a mouse lipocortin (Errasfa & Russo-Marie, 1989).

Glucocorticoids are also immunosuppressive, causing a reduction in the T and B cell population in the circulation (Axelrod et al., 1981). It is thought that glucocorticoids suppress the immune response by inhibiting cytokine production (Besedovsky et al., 1986). Interleukin-2 production, is suppressed by dexamethasone acting at the level of gene transcription (Gillis et al., 1979; Ariya et al., 1984). Synthesis of the cytokine interleukin-1 (IL-1) is also inhibited by glucocorticoids *in vivo* (Gander et al., 1968) and *in vitro* (Cunha et al., 1985). IL-1 has been implicated in the pathophysiology of arthritis (Bunning et al., 1986) and is found to release collagenase (Golds et al., 1983), promote bone resorption (Gowen et al., 1983), prostaglandin E₂ production (Mizel et al., 1981), neutrophil

degranulation (Kampschmidt et al., 1976) and release of oxygen-free radicals (Klempner et al., 1979). Peritoneal macrophages collected from rats following ADX, release more IL-1 than those from sham operated animals (Perretti et al., 1989). It is likely that the anti-inflammatory and immunosuppressive actions of glucocorticoids contribute to their efficacy in the symptomatic relief of inflammatory disorders.

1.5 AIMS

In this Chapter, I have described the possible mechanisms by which glucocorticoids may be useful in the treatment of painful and inflammatory conditions. The neuropeptides SP, SS and CGRP are found in the *drg*, and their putative role in the pathology of inflammatory pain has also been discussed. It may be that part of the action of glucocorticoids as anti-inflammatory analgesics involves regulation of SP, SS and CGRP in the *drg*. Consequently, in this thesis, I have investigated the effects of ADX and corticosteroids on the SP, SS and CGRP content of *drg* in the rat, both *in vivo* and *in vitro* using primary culture of adult rat *drg* enriched for neurons. Furthermore, I have assessed the changes in SP, SS and CGRP content of *drg* in a rat model of inflammatory disease, and determined the effects of ADX and glucocorticoids on the inflammation and changes in SP and CGRP content of *drg* in this model. Finally, I have investigated if another steroid, testosterone, also regulates neuropeptide content of *drg* in the rat, and described the distribution of the three neuropeptides under study in *drg* from level C1 to L6.

CHAPTER 2

GENERAL METHODS SECTION

2.1 SURGICAL PROCEDURES

2.1.1 Animals

In all experiments, male Ham Wistar rats (Charles Rivers, UK) were used. The animals were housed four to a cage in raised lid RP2 cages, and given grade 6 sawdust bedding and paper wool nesting material. Cages were cleaned daily and fresh food, water and bedding provided. Food (a CRM cubed diet; Special Diet Services, UK) and tap water were provided *ad libitum* and the temperature was thermostatically controlled between 68 and 72°F. Illumination was also automatically controlled, giving 14h of light between 5 a.m. and 7 p.m.

2.1.2 Anaesthetic

For surgical procedures, animals were anaesthetised under halothane (Fluothane, ICI) in oxygen (2.5l/min). For induction, 2% halothane concentration was used and animals were subsequently maintained in 1-1.5% halothane. Animals to be anaesthetised were placed in a perspex box 4" x 5" x 9" with a close-fitting lid and the box connected in series with a gas line from the anaesthetic machine (King Portable Boyles BOC Ltd.) fitted with a fluotec vapouriser (BOC Ltd.). A controlled O₂ flow could be switched into the anaesthetic supply at will, from 2 x 45lb medical oxygen cylinders carried on the apparatus. The exhaust gas from the induction box was led through rubber tubing into a bottle containing liquid paraffin through which it was bubbled, to remove most of the halothane before venting it to the operating theatre extraction system. Once induced, the animals were transferred to a 'small animal' face mask connected in series with the box, and received a maintenance dose of anaesthetic under which surgery was performed.

2.1.3 Adrenalectomy

2.1.3.1 Two Step

Animals were anaesthetised under halothane as described, and the fur removed from their flank using a small animal electric razor. The area was sterilised with ethanol and a small incision of approximately 1cm was made in the flank, 1.5cm from the spinal column, of the animal, the muscles were separated and the kidney located. The adrenal gland, lying above the kidney, was identified and excised with surrounding connective tissue. Care was taken to ensure that no fragments of the adrenal gland remained. The incision was sutured with running stitches and the animals placed in a 37°C incubator to recover. For the first ten post-operative days, the animals' diet was supplemented with a mash consisting of milk and brown bread, but subsequently they were fed on normal diet. Chlortetracycline (Aureomycin 10g/l; Cyanamid, UK) was included in the drinking water for the first ten post-operative days. Seven days later, the procedure was repeated, and the second adrenal gland removed. Bilateral adrenalectomised animals were given a choice of drinking water containing 1% saline (NaCl; BDH), 5% glucose (BDH) and chlortetracycline or tap water containing chlortetracycline. To verify successful adrenalectomy, plasma samples were collected on the termination of experiments and assayed for corticosterone and ACTH using RIA (see below). In addition, the cadaver was inspected post-mortem for adrenal fragments.

2.1.3.2 One Step

Animals were anaesthetised in halothane and both adrenal glands removed as described above. Following surgery, the animals' diet was supplemented as above, and they were given a choice of tap water or 1% saline, 5% glucose, supplemented with chlortetracycline.

2.1.3.3 Sham Operation

For sham operation, the animals were prepared and anaesthetised as for adrenalectomy. Incisions were made in the flanks, the muscle separated and the adrenal glands located, but not manipulated. Post-operative treatment was as for adrenalectomised animals.

2.1.4 Castration

Animals were anaesthetised under halothane anaesthesia and the scrotum swabbed with ethanol to sterilise the area prior to surgery. An incision was made in the tip of the scrotum to permit extrusion of the testis and a single ligature (2/0 silk) secured around the internal spermatic vessels, the deferential vessels and the *vas deferens* to minimise bleeding and facilitate excision of the testis. The testis, together with the caput, and caudate epididymes were excised by cutting above the ligature and the scrotum was sutured with one or two running stitches using 2/0 silk. The procedure was then repeated for the other testicle. For sham operation, animals were swabbed and the internal spermatic vessels, the deferential vessels and the *vas deferens* clamped; no incision was made into the testicle. For implantation of capsules, a small incision was made in the shoulder and using a pair of forceps a space was formed below the skin of the right upper forelimb. The capsule, either empty or containing testosterone, was then eased in under the skin and the incision stitched with two running sutures using 2/0 silk. Following surgery, animals were placed in an 37°C incubator to recover, and their diet was supplemented with a brown bread and milk mash. In addition, operated animals were given chlortetracycline (10g/l) in their drinking water.

2.1.5 Induction of Adjuvant Arthritis

Animals were anaesthetised with halothane and injected subdermally in the left forepaw with Freund's complete adjuvant (1mg/ml; Sigma, UK). Two injections of 50µl each were made at two separate locations in the forepaw. In some cases, control animals were given subdermal injections of incomplete adjuvant (Sigma, UK) in the left forepaw (two injections of 50µl each were administered).

2.1.6 Dissections

2.1.6.1 C4-C7

Animals were decapitated through the brain stem using a small animal guillotine and the dorsal surface of the spinal column exposed. The *drg* were exposed by laminectomy. Briefly, the spinous processes were removed from the dorsal surface of the spinal column using a large pair of rongeurs and subsequently a pair of fine rongeurs were used to remove the remainder of the dorsal surface of the vertebrae, the lamina (that part lying above the transverse processes), from the severed end to approximately level T3. The spinal cord thus exposed, was carefully excised using curved watchmaker's forceps (No. 7) and curved microdissection scissors (Weiss) to free the connective tissue attaching it to the ventral surface of the spinal column. The dorsal and ventral roots were also severed. Once the spinal cord was removed, the *drg* were visible lying on either side of the spinal column and the *drg* from level C4-C7 were identified by counting down from level C1. The *drg* were collected with the aid of a binocular dissecting microscope by grasping them firmly with fine forceps and severing the dorsal root and peripheral nerve with curved microdissection scissors. They were collected in bilateral pairs in Eppendorf tubes, frozen on dry ice and stored at -20°C until processed. With practice, this procedure could be completed in 3-4 mins from the time of decapitation.



2.1.6.2 C2/3-T5/6

The animals were decapitated and *drg* exposed by laminectomy as described above, from the severed end to approximately level T8. Identified *drg* were collected unilaterally in pairs C2/3, C4/5, C6/7, T1/2, T3/4 and T5/6 from the left and right sides. They were placed in Eppendorf tubes, frozen on dry ice, and stored at -20°C until processed. This procedure typically took around 10 mins from the time of decapitation.

2.1.6.3 C1-L6

Animals were killed by asphyxiation in a high CO₂ atmosphere. This was achieved by placing the animal in a dessicator with dry ice in the bottom and replacing the lid, sleep and death typically ensued within 1 min. The dorsal surface of the spinal column was exposed by making an incision from the head to the base of the tail on either side of the spinous processes. Skin, overlying flesh, and spinous processes were removed by using a large pair of rongeurs and the bone was freed from above the cerebellum. The dorsal surface of the spinal column was removed by laminectomy as described above, from the atlas to below L6. Following removal of the spinal cord, *drg* were removed bilaterally in pairs from C1 to L6, frozen on dry ice and stored at -20°C until processed.

2.1.7 Collection of plasma samples

Trunk blood samples were collected following decapitation. Animals were allowed to bleed through a filter funnel into plasma tubes held on ice. Prior to collection, the funnel and tubes were washed in 0.9% saline containing 100U/ml of Heparin (Evans Medical, UK) and 1000KIU/ml of Trasylol (Bayer, UK). Immediately following collection, the tubes were inverted a number of times and then centrifuged at 2300 x g for 30 min at

4°C to pellet the cells. The supernatant was removed with a glass pipette and stored in Eppendorf tubes at -40°C until assayed.

2.1.8 Preparation of *drg* samples for RIA

Drg were homogenised in 100µl 2M acetic acid using 0.1ml rod formed glass mini homogenisers (Jencons, UK) and centrifuged at 16000g for 10 mins. The supernatant was removed and stored at -20°C prior to assay.

2.1.9 Drugs

2.1.9.1 Injections

Injections were administered subcutaneously into the scruff of the neck.

Corticosterone (B; Sigma, UK)

Stock concentration of 2.5mg/ml in sesame oil was given daily in volume of 200µl to a final dose of 2.5mg/kg.

Dexamethasone (DEX; Sigma, UK)

Stock concentration of 0.2mg/ml in sesame oil administered daily in 200µl volume giving a final dose of 0.2mg/kg.

RU38486 (Roussel UCLAF, France)

Stock concentration of 10mg/ml in sesame oil given daily in 200µl injection at a dose of 10mg/kg.

Control Animals

Control animals received daily injections of 200µl sesame oil.

2.1.9.2 Oral Dosing

Drugs were administered orally by gavage, using a round end needle.

DEX (Sigma, UK)

Stock concentration of 0.05mg/ml in 0.9% saline, 0.2% ethanol administered twice daily by gavage in 0.4ml/100g volume giving a final dose of 0.2mg/kg.

RU38486 (Sigma, UK)

Stock concentration of 2.5mg/ml in 0.9% saline, 0.2% ethanol administered twice daily by gavage in 0.4ml/100g volume giving a final dose of 10mg/kg.

Control Animals

Controls received 0.4ml/100g of 0.9% saline, 0.2% ethanol.

2.1.9.3 Pellets

Reagents: Corticosterone (Sigma, UK)

Cholesterol (Sigma, UK)

50% w/w of B/cholesterol was mixed and heated rapidly in an oil bath at 260°C until melted. The molten mixture was then poured into the moulds used for mounting samples for electron microscopy until full, which gave pellets of 200mg. They were allowed to cool, at which time the pellets could be easily removed. For control animals, pellets of the same shape and size were cut out of bone wax. Pellets were implanted subcutaneously in the scruff of the neck and replaced after 15 days.

2.1.9.4 Silastic Capsules

Silastic capsules were used to administer testosterone to castrated animals. A 30mm length of silastic tubing (Dow Corning Corp., Michigan, USA) of internal diameter 1.57mm and external diameter 3.18mm was filled with crystalline testosterone propionate (Sigma, UK) and sealed with wax. Prior to implantation, they were soaked in 0.9% saline at room temperature to ensure they contained no leaks.

2.2 PRIMARY CULTURE

2.2.1 Tissue Culture Plastic

Tissue culture plates used in these experiments were:

35mm x 15mm - Falcon 3001F

60mm x 15mm - Falcon 1007

140mm x 17mm - Sterilin 501V

35mm x 15mm, 4 raised ring dishes - CelCult

2.2.1.1 Poly-Ornithine Coating (Collins, 1978)

Reagent: Poly-DL-Ornithine Hydrobromide (Sigma, UK).

Polyornithine was dissolved in 0.15M $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ /0.15M H_2BO_4 , pH 8.3 to final concentration of 500 $\mu\text{g}/\text{ml}$ and filter sterilised through a 0.2 μm filter. To coat 60mm plates, 6ml of this solution was placed in each plate and left at room temperature for between 6 and 18 hrs. The solution was aspirated from the plate and it was washed twice in sterile distilled water. For four-well plates 80 μl per well of polyornithine was used; after incubation, the solution was aspirated and the plates washed twice in sterile distilled water.

2.2.1.2 Laminin Coating (Edgar et al., 1984)

Reagent: Laminin (1.15mg/ml; NBL, USA)

Laminin was dissolved in sterile Dulbecco's phosphate buffered saline to concentration 5 $\mu\text{g}/\text{ml}$. To each of the four wells of previously polyornithine-coated plates 80 μl of the laminin solution was applied and incubated at room temperature for at least two hours. The solution was aspirated and the wells washed twice in phosphate buffered saline.

2.2.2 Incubator

Cells were incubated at 37°C in 3% CO₂ atmosphere at 100% humidity. The incubator was a water jacketed Forma-Scientific model 3157 (Forma Scientific, Marietta, Ohio USA).

2.2.3 Culture Medium

Reagents: Hams Nutrient Mixture F14 - Imperial Laboratories (see Appendix A for composition)
L-Glutamine (200mM) - Flow Laboratories
Penicillin (100IU/ml)/Streptomycin (100µg/ml) - Gibco
Na HCO₃ - BDH

Hams Nutrient Mixture F14 was dissolved in Millipore filtered water (12.11g/l) and Na HCO₃ (1.176g/l) and L-glutamine added (5ml). The solution was sterilised through a 0.22µm bottle top filter (Falcon, 1705), attached to a water pump and penicillin and streptomycin added (1:100 dilution); the solution was referred to as F14 basic medium. Medium was stored at 4°C but was incubated for 1h at 37°C prior to use in order to reduce the cold shock for cells. Medium was never used over one month old due to loss of NaHCO₃ and L-glutamine from the medium.

2.2.4 Drugs and Supplements

Ultra Ser 'G' (Gibco, UK)

Ultra Ser 'G', a synthetic serum, was purchased as a lyophilised powder in sealed sterile glass bottles. The powder was reconstituted under aseptic conditions in 20ml F14 basic medium and the dissolved product was added to 500ml basic medium, giving a final concentration of 4% v/v.

Cis-4-OH Proline (Sigma, UK):

Cis-4-OH proline was dissolved in F14 basic medium and filter sterilised to give a final stock concentration of 20mg/ml. The stock was

stored at 4°C, and 25µl added to each 35mm plate (containing 2.5ml medium) to give a final concentration of 200µg/ml.

Nerve Growth Factor (NGF; kind gift of R.M. Lindsay):

NGF was received at a concentration of 100mg/ml and diluted further in F14 basic medium giving a stock concentration of 2.5µg/ml. It was stored at 4°C and 10µl was added to each 35mm plate giving a final plate concentration of 25ng/ml.

B (Sigma, UK)

The powder was dissolved in ethanol to a stock concentration of 10mg/ml which was stored in a foil-wrapped tube at 4°C. 25µl was added to each plate, giving a final concentration of 100µg/ml.

DEX (Sigma, UK)

Dexamethasone was dissolved in ethanol to give a stock concentration of 1mM. This was stored in a foil-wrapped tube at 4°C and 25µl added to each plate giving a final concentration of 10µM.

Forskolin (Sigma, UK)

Forskolin was dissolved in tissue culture grade dimethylsulfoxide (Sigma, UK) to stock concentration 800µM and stored at 4°C. 25µl of this stock was added to each plate, giving a plate concentration of 8µM.

ITS+ (Collaborative Research Ltd., UK)

ITS+ was received as a concentrated aqueous solution containing insulin (12.5mg), transferrin (12.5mg), selenic acid (12.5mg), bovine serum albumin (2.5g) and linoleic acid (10.7mg) in a volume of 20ml. The stock solution was diluted 1:100 in F14 basic medium to give final concentrations as below:

Insulin 6.25 μ g/ml

Transferrin 6.25 μ g/ml

Selenic Acid 6.25 μ g/ml

Bovine serum albumin 1.25mg/ml

Linoleic acid 5.35 μ g/ml

2.2.5 Dissection

All dissection instruments were placed in 70% ethanol in water for at least 15 minutes prior to commencing dissection, which was performed on the open bench. Periodically throughout the dissection, the instruments were resterilised in 70% ethanol. The animals (Ham Wistar rats, male, 200g) were decapitated and the cadaver sprayed with 70% ethanol to sterilise the outer surface. The dorsal surface of the animal was exposed using a sterile scalpel blade and tissue overlying the spinal cord removed with a large pair of dissection scissors. Starting at the severed end, the spinal column was excised from the carcass by cutting the muscle free from either side of the column and freeing the outer ventral surface from the underlying connective tissue. Once free, the cord was transferred to a sterile 140 x 17mm tissue culture dish, and using a small pair of scissors, an incision made down the length of the dorsal surface of the column on either side of the spinous processes. The resulting strip of bone was removed and the two halves of the column split by cutting along the midline on the ventral surface. Treating each side separately, the overlying spinal cord was removed with the aid of a binocular microscope and *drg* teased free by grasping the dorsal root firmly between watchmaker's forceps (No. 7) and cutting this root and the peripheral root as near to the *drg* as possible. *Drg* were collected in F14 basic medium containing 4% USG, and freed of connective tissue and remnants of dorsal roots. Typically, 40-45 *drg* were collected and cleaned from each animal in approximately 1h 20 min.

2.2.6 Plating Procedure

2.2.6.1 Enzyme Treatment

10 x stock solutions: 5% Collagenase (Worthington, UK) in F14 medium containing 4% USG: 2.5% Trypsin (Worthington, UK) in F14 medium.

Stock solutions were stored in 400µl aliquots at -70°C.

Procedure

Cleaned *drg* were transferred to a laminar flow hood in a 35 x 15mm dish and the medium removed with a sterile glass pipette. An aliquot of the 10 x stock collagenase was thawed, diluted 1:10 in F14 containing 4% USG and filter sterilised. The *drg* were then placed in this solution and incubated for 3h at 37°C. Following incubation, the *drg* and medium were removed from the dish using a 10ml sterile disposable pipette and placed in a sterile 10ml tube. The *drg* quickly settled to the bottom of the tube and were washed twice in 10ml F14 basic medium. An aliquot of 10 x stock trypsin was diluted 1:10 in F14 basic medium and the *drg* incubated in this solution for 30 min at 37°C. Following incubation, the *drg* were washed twice in 10ml F14 containing 4% USG and resuspended in 2ml of F14 containing 4% USG.

2.2.6.2 Trituration

The tip of a Pasteur pipette was fire-polished in a bunsen flame until the orifice was typically between 0.5mm and 1mm in diameter (Lindsay, 1988). Digested *drg* contained in 2ml of F14 containing 4% USG were taken up into the pipette and gently passed out. This procedure was repeated until the majority of *drg* were dissociated, and a milky solution was obtained.

2.2.6.3 Neuronal enrichment and plating

The solution containing dissociated *drg* was diluted such that it was contained in 4mls F14/4% USG for each rat that was dissected. Following mixing in a pipette to produce a homogenous solution, 2ml of this solution was added to an appropriate number of 60mm culture dishes precoated with poly-ornithine. The solution was agitated to cover the whole plate and a further 2ml F14 containing 4% USG added, before they were placed in the incubator for between 15 and 20h (typically 18h).

Following incubation, the medium was removed, using a fire-polished pipette attached to a Buchner vacuum pump, and the plates were washed twice with 3ml F14/4% USG. The neurons which adhered loosely to the plate were detached by a gentle stream of medium from a fire-polished pipette and collected from each plate in 2ml F14 4% USG leaving the more strongly adherent non-neuronal cells on the plate. Further purification was achieved, due to the differential sedimentation of neurons and non-neuronal cells, by centrifugation for 5 min at 680 x g. The supernatant was removed and the pellet, containing the neurons, resuspended in 1ml of F14/4% USG. Cells were counted in a haemocytometer and resuspended in F14/4% USG to give a final concentration of between 3000 and 5000 cells per 80 μ l medium. The solution was gently mixed to ensure the cells were evenly distributed and the cells plated onto laminin-coated 35mm 4 well dishes. 80 μ l was added to each well, and the plates transferred to the incubator for 3-4h to allow the cells to adhere. Following this settling period, the plates were flooded with medium to a final volume of 2.5ml. Medium was supplemented with cis 4-OH proline (25 μ l, stock solution to final concentration 200 μ g/ml) to inhibit non-neuronal cell proliferation, and was changed every two days.

2.2.7 Sample extraction

The samples were removed from the wells in 2M acetic acid. The medium was aspirated from the plates, 60 μ l 2M acetic acid added to each well, the bottom of the well scraped with a pipette tip and the plates incubated for 30 min at 37°C. The solution was removed, collected in Eppendorf tubes and centrifuged for 5 min at 16000g in an Eppendorf bench centrifuge; the supernatant was removed and stored at -20°C until assayed.

2.3 RADIOIMMUNOASSAY

2.3.1 General Methodology

2.3.1.1 Introduction

Radioimmunoassay (RIA) is a well-established technique used in many laboratories throughout the world to measure the concentrations in blood, tissues and other fluids of peptide and non-peptide hormones, steroid hormones, drugs and other substances of biological interest. The method can be expeditious, highly sensitive and relatively specific. RIA is based on a concept developed in the late 1950s by Yalow & Berson to measure insulin in the plasma of patients (Yalow & Berson, 1960). Independently, around the same period, Ekins (1960) developed a method for measuring serum thyroxine levels based on the same principles as RIA, but relying on the naturally occurring binding protein thyroxine binding globulin rather than an antibody as the specific reagent in this 'saturation assay method'.

The principles underlying RIA are relatively simple, based on the law of Mass Action. The concentration of an unknown sample is obtained by comparing its inhibitory effect on the binding of radioactively labelled antigen or tracer to a specific antibody with that of a series of known, standard concentrations of antigen (see Ekins, 1974; Yalow, 1980).

In order that the binding characteristics of both standards and unknown are the same, the solutions should be identical in all respects,

excepting the concentration of ligand. In practice, this is not always achieved, as it is not possible to produce, synthetically, the environment of tissue extract, and consequently non-specific effects must be considered. If the standard curve is parallel to a dilution curve of sample extract, this is usually considered sufficient indication that the binding characteristics of both standard and sample solutions are similar under the assay conditions.

Non-specific binding of the antibody may also produce specious results. Clearly a tissue extract contains a large number of proteins and peptides and it is possible the antibody may have low affinity for one or more of these non-specific ligands. This non-specific binding is detectable by a departure from parallelism between the standard curve and a dilution curve of sample. Figs. 2, 2 and 2.5 show the parallelism between standard dilution curves and sample dilution curves for the SS, SP and CGRP RIAs used. In addition, the cross-reactivity of each assay for the other two ligands was determined.

2.3.1.2 Preparation of Antisera

Antibodies are produced in an animal when the cells of the immune system encounter a foreign compound, be it protein, polysaccharide or DNA. Generally, a molecule only stimulates an immune response if it is over 10,000 daltons in molecular weight. However, many compounds of interest, including SS, CGRP and SP, are less than this minimum weight. In order to raise antisera to smaller molecules, they first have to be coupled to a larger molecule known as a carrier protein.

2.3.1.3 Antisera

The anti-SS and anti-SP antisera were raised by Dr. A.J. Harmar and Dr. P. Keen in Bristol (Harmar & Keen, 1976), using a method based on that described by Carraway & Leeman (1976) for raising antisera against

neurotensin. SP and SS both have molecular weight below 10,000 daltons and thus were conjugated to succinylated thyroglobulin. Forty milligrams of bovine thyroglobulin (Sigma, UK) was dissolved in 8ml 0.15M NaCl, and the pH adjusted to 7 with Na_2CO_3 . Over the space of 1h, 80mg of solid succinic anhydride was added to this solution with the pH being maintained at 7 by further addition of Na_2CO_3 . The solutions reacted for a further 30 min and were then dialysed against distilled water and lyophilised. For conjugation of peptides, 20mg of the succinylated thyroglobulin was dissolved in 1ml of 0.1M phosphate buffer pH 7.4 and 10mg SP or 10mg SS-14 (UCB Bioproducts, Belgium) was added. The reaction was achieved by the dropwise addition of 10mg 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide-HCl (Sigma, UK: 20mg/ml in phosphate buffer, pH 7.4). This mixture was kept in the dark and stirred for 20h, dialysed for 48h against distilled water, and lyophilised.

To raise antisera using the resultant conjugates, the lyophilisate was suspended in 4.5ml 0.15M NaCl and emulsified with a further 4.5ml Freund's complete adjuvant supplemented with heat-killed tubercle bacilli (5mg/ml, Difco, USA). Two millilitres of the immunogen was administered by multiple intradermal injections to each of four New Zealand white rabbits together with a single s.c. injection of pertussis vaccine (Difco; 0.5ml). Following a booster injection 6 weeks later of a further 2ml immunogen, the animals were bled from the ear vein at 10-14 day intervals. The anti-SS antiserum and the anti-SP antiserum used in this thesis were obtained from single animals respectively seven and eight weeks after the booster dose.

2.3.1.3 Iodination

A number of methods are available for the radioiodination of peptides and proteins, which can be divided into direct methods in which ^{125}I is incorporated directly into tyrosine and/or histidine residues, and conjugation

methods in which a radioiodinated moiety is conjugated to a specific side chain usually ϵ -NH₂ groups of lysine residues or at the N-terminus. Whilst conjugation is more complex, it has the advantage that it can be used to label peptides that do not contain tyrosine residues.

The most widely used method of iodination is the chloramine-T oxidation of Na¹²⁵I in the presence of the protein or peptide to be labelled (Greenwood & Hunter, 1962). This results in incorporation of ¹²⁵I into the tyrosine residues in high yield but, unfortunately, the presence of oxidising agents can also damage the peptide. Whilst peptide damage can be reduced by addition of a reducing agent such as sodium metabisulphite to remove excess chloramine-T, the use of a solid phase oxidising agent is more satisfactory. 1,3,4,6-tetrachloro-3 α , 6 α -diphenylglycoturil (Iodogen, Fraker & Speck, 1978) is coated onto the surface of the reaction vessel and iodination affected by the addition of Na¹²⁵I and peptide solution. A further, more gentle, method was first described by Marchalonis (1969) in which iodination of peptide is catalysed by lactoperoxidase in the presence of small amounts of hydrogen peroxide.

2.3.2 Radioimmunoassay for Somatostatin

2.3.2.1 Buffers

The standard assay buffer consisted of 0.1M sodium phosphate pH 7.4 with 0.15% w/v BSA. Antiserum buffer comprised 0.1M sodium phosphate, pH 7.0, 0.1% w/v BSA, 0.1% w/v Triton X-100, 250KIU/ml Trasylol (Bayer).

2.3.2.2 Standard Curve

A stock solution of SS-14 (Peninsula) of 10 μ g/ml in 0.1M acetic acid, 0.1% w/v BSA was prepared and stored at -40°C. Aliquots of the stock solution were evaporated to dryness, resuspended in assay buffer and a

series of dilutions in assay buffer, prepared in duplicate, of 10, 20, 35, 50, 70, 100, 140 and 200pg/ml in 100µl volume. The standard curve was constructed by an on-line computer using a best fitting linear regression programme and a log-logit transformation. Unknowns were compared automatically by the computer (see Fig. 2.1).

2.3.2.3 Primary Antibody

The antibody was raised in rabbit against SS-14 conjugated to succinylated bovine thyroglobulin (Harmar & Keen, 1976). Stock antiserum was kept at -40°C and diluted to 1/200000th strength in antiserum buffer and 100µl added to all assay tubes except total counts and non-specific binding tubes.

2.3.2.4 Labelled Antigen

Antigen was labelled using the iodogen method (Fraker & Speck, 1978). Iodogen (diphenylglycoturil) was coated onto the surface of the reaction vessel and iodination affected by the addition of Na¹²⁵I and peptide solution. The reaction was stopped by dilution with 0.05M sodium phosphate and potassium iodide to the mixture.

Solutions

Tyr^o SS-14 - Peninsula, 0.25mg/ml in 5mM phosphate buffer, pH 7.4

Na¹²⁵I - Amersham (1MS 30): 3.7GBq/ml

Iodogen - 40µg/ml in dichloromethane

Phosphate buffer - 0.05M pH 7.4

KI - 10µg/ml in aqueous 0.2% v/v TFA

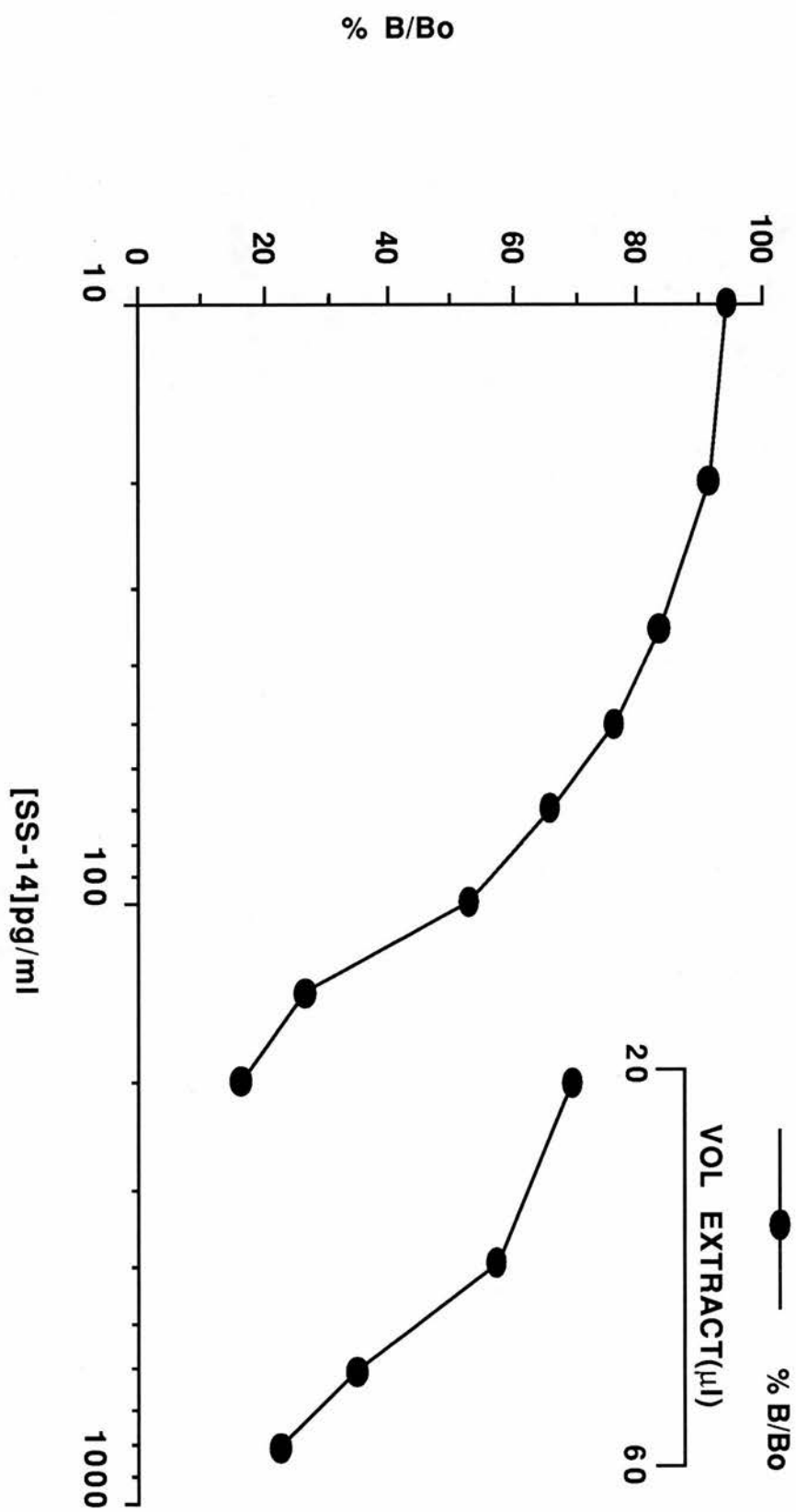
Method:

50µl Iodogen was evaporated to dryness under nitrogen in a polypropylene reaction vessel. 5µg Tyr^o SS-14 (20µl of stock solution) and 10µl (1mCi) Na¹²⁵I were added, mixed and allowed to react for 10 min.

Figure 2.1

Graph showing a typical computed standard dilution curve for SS-14. The dilution curve of SS content of homogenised *drg* is also shown

Fig. 2.1. SS-14 RIA standard curve and sample dilution curve

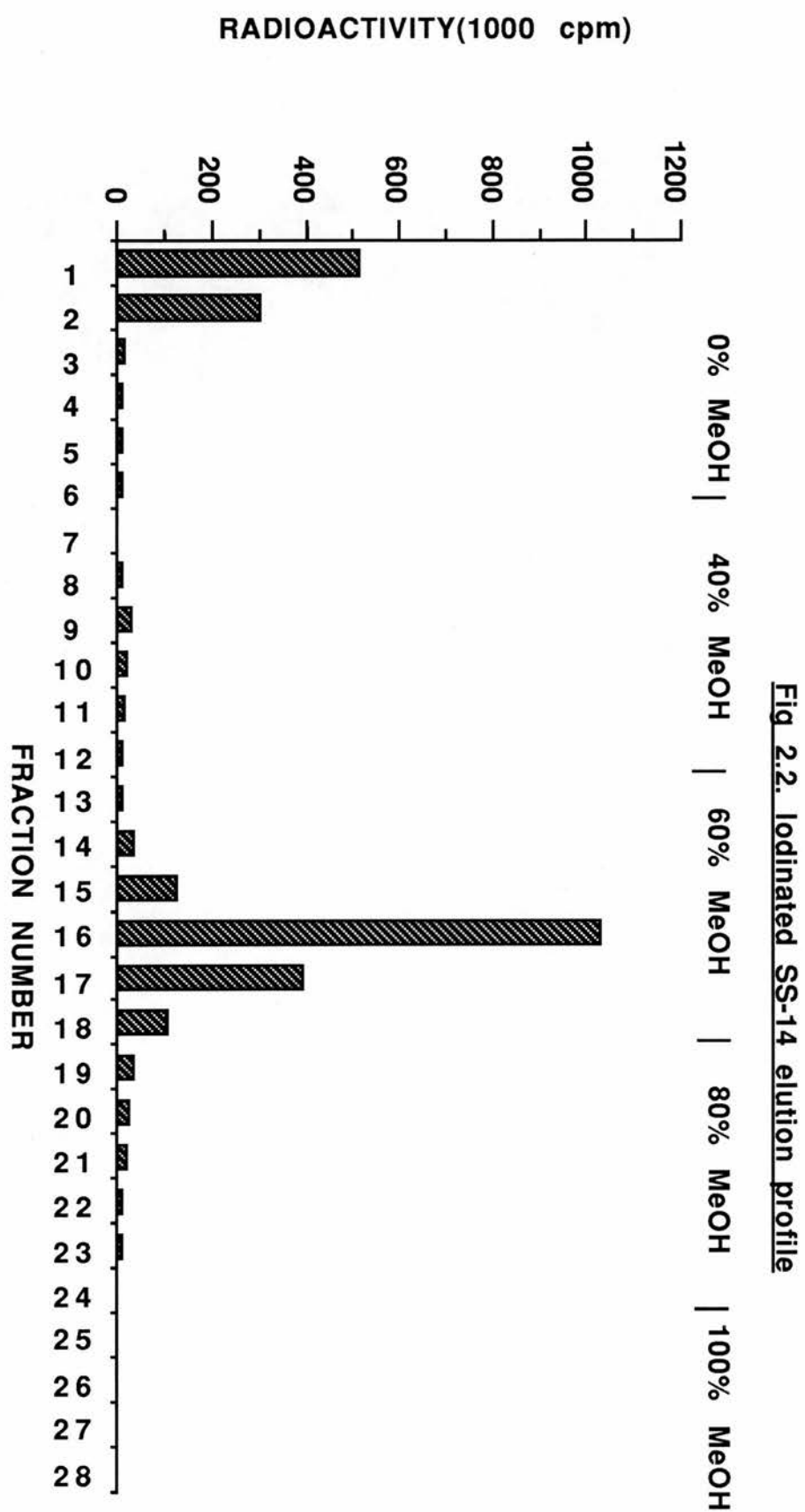


The reaction was interrupted by addition of 200 μ l 0.05M phosphate buffer and 500 μ l KI solution. Labelled peptide was then separated from unreacted iodide and peptide by HPLC (Harmar & Rosie, 1984). The chromatography system comprised a two-channel minipump (Milton Roy Co., Riviera Beach, Fl., USA) connected in series to a sample injection valve with a 1ml loop (Rheodyne Berkeley, CA, USA) and a guard column holder (Brownlee MPLC Cartridge system: Brownlee Labs, Santa Clara, CA, USA). Usually, a 3cm x 0.46cm disposable column of cyanopropyl silica (Spheri 5 cyano: Brownlee Labs) was used, although in some cases a similar sized column of C₁₈ silica (RP-18 Spheri 5: Brownlee Labs) was used with comparable results. The two channels were primed respectively with 0.2% aqueous TFA (solvent A) and 0.2% TFA in methanol (solvent B). Following equilibration of the column with solvent A, the iodination mixture was loaded and eluted with a stepwise gradient of solvent B from 0%-100%. The flow rate was 1ml/min and six fractions of 1ml were collected at each step in the gradient. The elution profile of ¹²⁵I-labelled Tyr^o SS-14 which eluted in 60% methanol is shown in Figure 2.2. Fractions 16 and 17 were combined in a further 2ml of 0.2% TFA in methanol and stored at -70°C prior to use for RIA.

2.3.2.5 Second Antibody

Bound, labelled antigen was separated from unbound labelled antigen by precipitation using donkey anti-rabbit gamma globulin (ARGG; Scottish Antibody Production Unit, Carlisle; SAPU). 100 μ l ARGG in assay buffer (1:10 dilution) was added and to reduce background binding 100 μ l normal rabbit serum (1:100 NRS; SAPU) was added.

Figure 2.2 Typical elution profile of ^{125}I -labelled Tyr^o SS-14 from a 30 x 4.6mm cyanopropyl silica column with increasing methanol concentration. Fractions 16 and 17 were pooled and stored at -70°C prior to use in RIA



2.3.2.6 Pre-spin Wash

Assay tubes received 1.5ml of assay buffer before centrifugation to reduce slippage of the precipitate caused by the previous addition of Triton-X-100 in the antiserum buffer.

2.3.2.7 Quality Control

Pools were added at the start and end of assays to serve as indicators of intra assay consistency and for comparison between assays. The pools were derived from previously assayed tissue samples and contained 30pg/ml and 140pg/ml respectively. For larger assays, additional pools were placed in the middle of the assay. The inter-assay CoV was < 20%, and intra-assay CoV was < 10%.

2.3.2.8 Cross-Reactivity

SS assay did not cross-react with CGRP (<0.1%) or SP (<0.01%) at the highest concentrations tested. The anti-SS serum was reportedly directed against the C-terminal or central portion of the SS-14 molecule as SS-28 and SS-25 are equipotent in displacing [125 I-Tyr^o] SS-14 from the antiserum (Harmar & Keen, 1986).

2.3.2.9 Assay Procedure

Day 1:

Samples in 2M acetic acid were evaporated to dryness in polystyrene RIA tubes (Luckham) in a vacuum oven (45°C, 700mm Hg) and resuspended in 240µl assay buffer. Duplicate samples of 100µl were prepared and 100µl of antiserum in antiserum buffer was added to unknowns, standards and residual binding tubes. Tubes for total counts received no solution and those for non-specific binding were given 100µl

assay buffer and 100µl antiserum buffers. The tubes were vortexed and incubated at 4°C overnight (minimum 16h).

Day 2:

100µl of [125 I] Tyr^o SS-14 diluted in antiserum buffer containing 5000cpm was added, vortexed and incubated at 4°C for a minimum 16h.

Day 3:

100µl ARGG and NRS was added, vortexed and the assay tubes incubated at 4°C overnight.

Day 4:

All tubes, except for the total counts tubes, received 1.5ml assay buffer and were centrifuged at 1,720 x g for 45 mins at 4°C. The supernatant was aspirated from the tube and the radioactivity remaining in the pellet was determined by counting in a gamma counter for 3 min.

2.3.3 Radioimmunoassay for Substance P

2.3.3.1 Buffers

The standard assay buffer consisted of 0.05M Na₂ HPO₄, 0.05M KH₂ PO₄, 0.14M NaCl, 0.05% w/v NaN₃, 0.1% w/v gelatin at pH 7.4.

2.3.3.2 Standard Curve

A stock solution of SP (Peninsula, 100µg/ml) in 0.2% aqueous TFA was prepared and stored at -40°C. Aliquots of this stock solution were evaporated to dryness, resuspended in assay buffer and a series of dilutions of 25, 50, 100, 250, 500, 1000, 2000 and 5000pg/ml in 200µl volume prepared in duplicate. The standard curve was constructed by an on-line computer using a best fit linear regression programme and a log-logit transformation. Unknowns were compared to the standard curve by the computer and expressed in pg/ml (see Fig. 2.3).

Figure 2.3

Graph of a typical standard dilution curve of SP. The dilution curve of SP content of homogenised *drg* is also shown

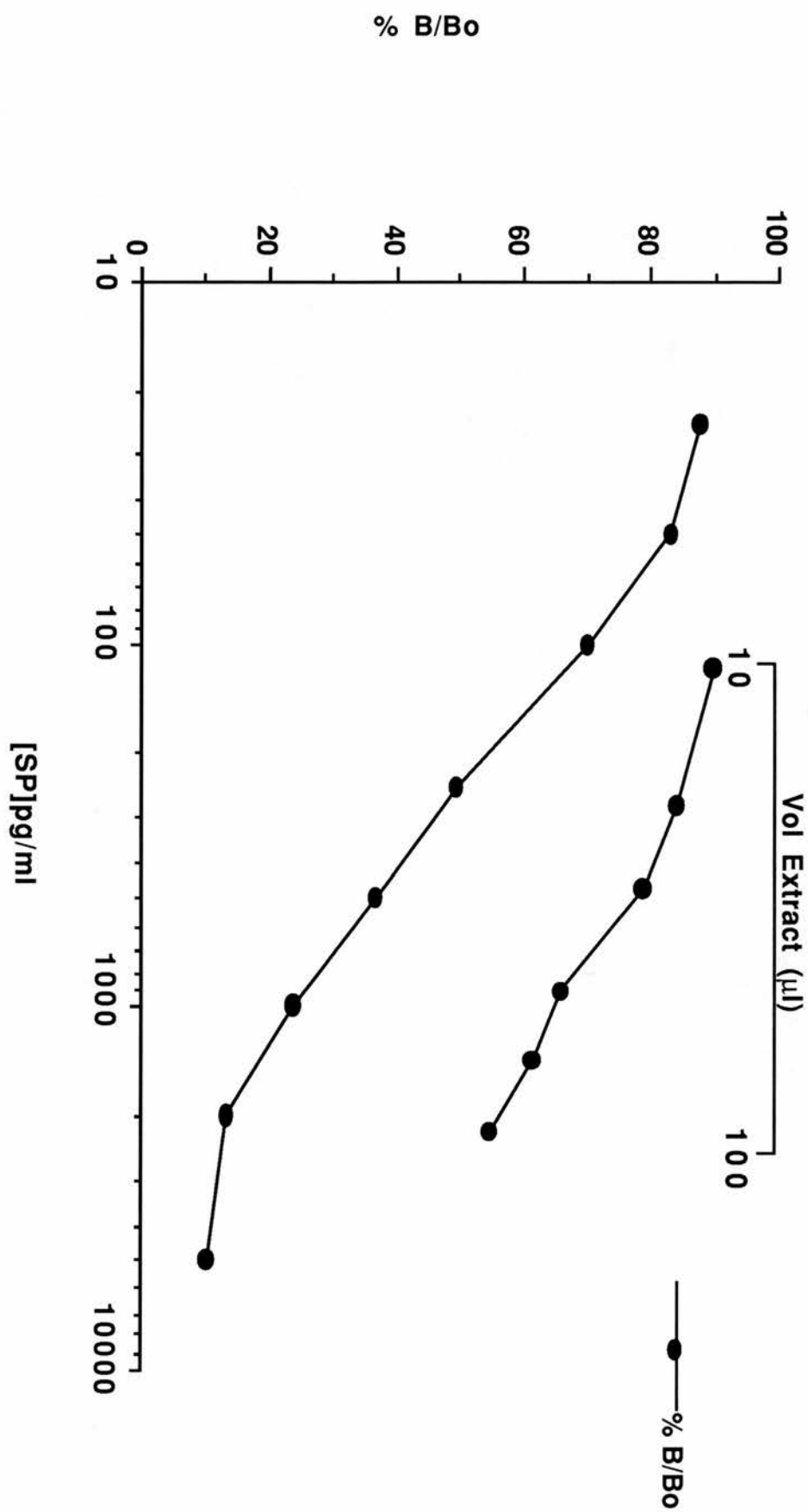


Fig 2.3. SP RIA standard curve and sample dilution curve

2.3.3.3 Primary Antibody

The antibody was raised in rabbit against SP conjugated to succinylated bovine thyroglobulin (Harmar & Keen, 1976). Stock antiserum was kept at -40°C and diluted 1:60000 in assay buffer. 100µl was added to all assay tubes except the total counts and non-specific binding tubes.

2.3.3.4 Labelled antigen

Tyr⁸ SP (Peninsula) was labelled using the iodogen method (Fraker & Speck, 1978).

Solutions

Tyr⁸ SP - Peninsula, 500µg/ml in 0.5M Na₂ PO₄, pH 7.4

Na¹²⁵I - Amersham 1MS 30: 3.7 G.Bq/ml

Iodogen - 40µg/ml in dichloromethane

Trifluoroacetic acid - 0.2% aqueous

Method:

50µl of iodogen was evaporated to dryness under nitrogen in a polypropylene reaction vessel. 5µg Tyr⁸ SP (10µl of stock solution) and 10µl (1mCi) Na¹²⁵I were added, mixed and allowed to react for 10 min at room temperature. The reaction was stopped by addition of 750µl, 0.2% aqueous trifluoroacetic acid. Unreacted iodide and peptide was removed by HPLC from the labelled peptide using HPLC as described in the iodination of SS-14 above. Briefly, the reaction mixture was loaded onto a 30mm x 4.6mm column of cyanopropyl silica and eluted with trifluoroacetic acid containing increasing amounts of methanol. The elution profile is shown in Fig 1.4; fractions 24 and 25 were pooled, diluted in a further 2ml of 0.2% TFA in methanol and stored at -70°C, prior to use for RIA.

Figure 2.4 Typical elution profile of ^{125}I -labelled Tyr⁸ SP from a 30 x 4.6mm cyanopropyl silica column, with increasing methanol concentration. Fractions 24 and 25 were pooled, diluted in a further 2ml of 0.2% TFA in methanol and stored at -70°C prior to use in RIA

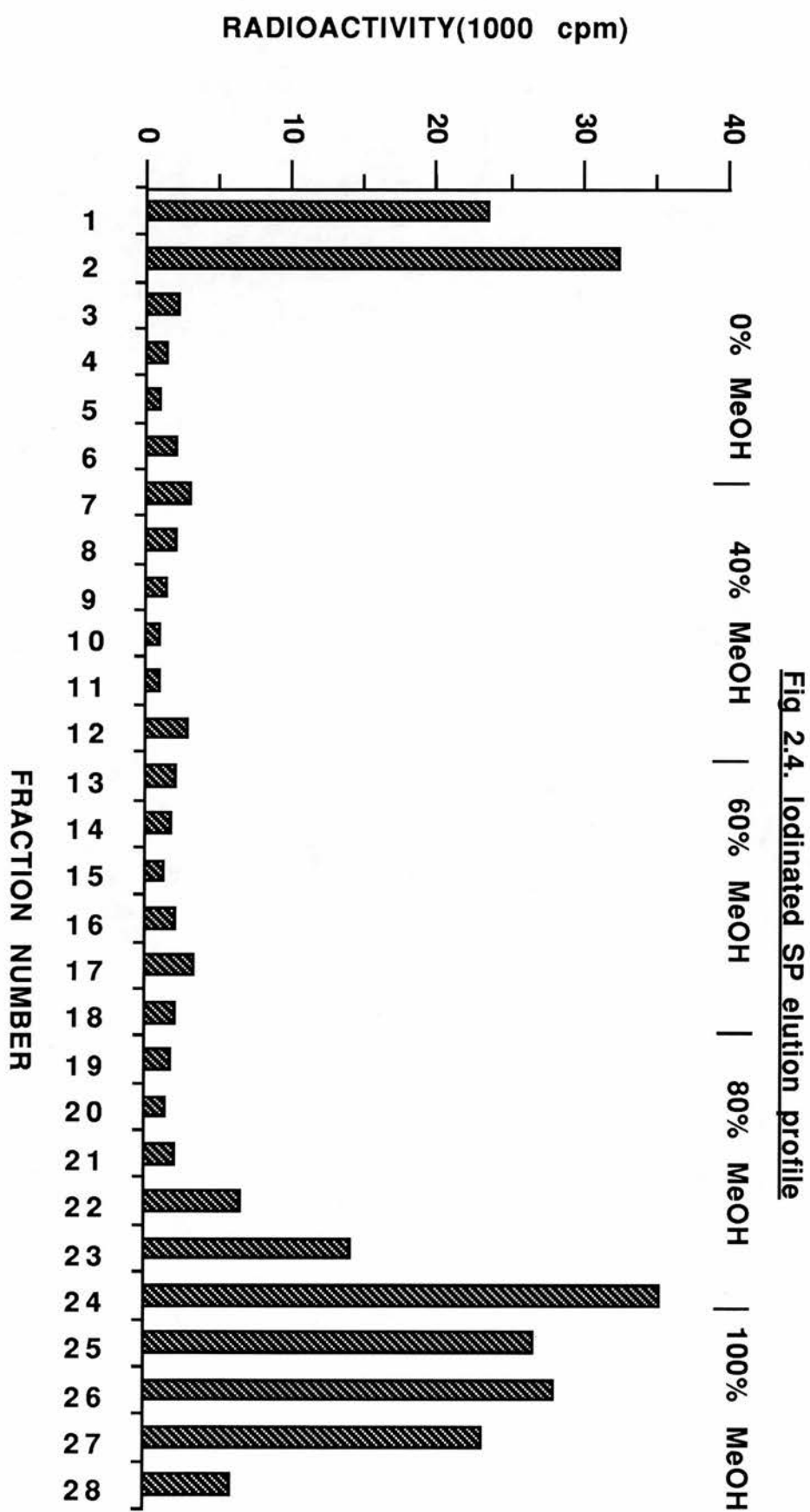


Fig 2.4. Iodinated SP elution profile

2.3.3.5 Second Antibody

Bound, labelled antigen was separated from unbound by precipitation using ARGG. 500µl of assay buffer containing NRS (1:1000 dilution) and ARGG (1:100 dilution) were added to all tubes except the total counts.

2.3.3.6 Quality Control

Pools were placed at the beginnings and ends of assays, and for larger assays, pools were added to the middle of the assay. The pools used were old standards and contained 70pg/ml and 800pg/ml respectively. Interassay CoV was <20% and intra-assay CoV was <10%.

2.3.3.7 Cross-Reactivity

The assay did not cross-react with SS-14 (<0.02%) or CGRP (<0.1%) at the highest concentrations tested (see Fig. 2.3). The anti-SP antiserum was reported to be specific for the C-terminal 5 amino acids of the SP sequence. SP free acid, lacking the C-terminal carboxamide has negligible affinity for the antiserum and NKA shows $1/70$ th the potency of SP (Harmar & Keen, 1986).

2.3.3.8 Assay Procedure

Day 1:

Samples in acetic acid were evaporated to dryness as for the SS assay and resuspended in assay buffer. 200µl aliquots of sample or standard were added to 100µl of antiserum, the tube mixed and incubated overnight at 4°C. Samples and standards were prepared in duplicate. Tubes for total counts received no solution and those for non-specific binding were given 300µl assay buffer.

Day 2:

100µl of labelled antigen in assay buffer was added to all tubes, the solutions mixed and incubated overnight at 4°C.

Day 3:

500µl of ARGG and NRS was added to all tubes except total counts, the tubes were mixed and incubated overnight at 4°C.

Day 4:

All tubes except the total counts were centrifuged at 4°C for 30 min at 1720 x g and the supernatants aspirated to leave the precipitate. The radioactivity in the pellet was counted for 3 min in a gamma counter.

2.3.4 RADIOIMMUNOASSAY FOR CGRP

2.3.4.1 Buffers

The standard assay buffer was 0.05M sodium phosphate, 0.05M sodium EDTA, 0.3% w/v BSA.

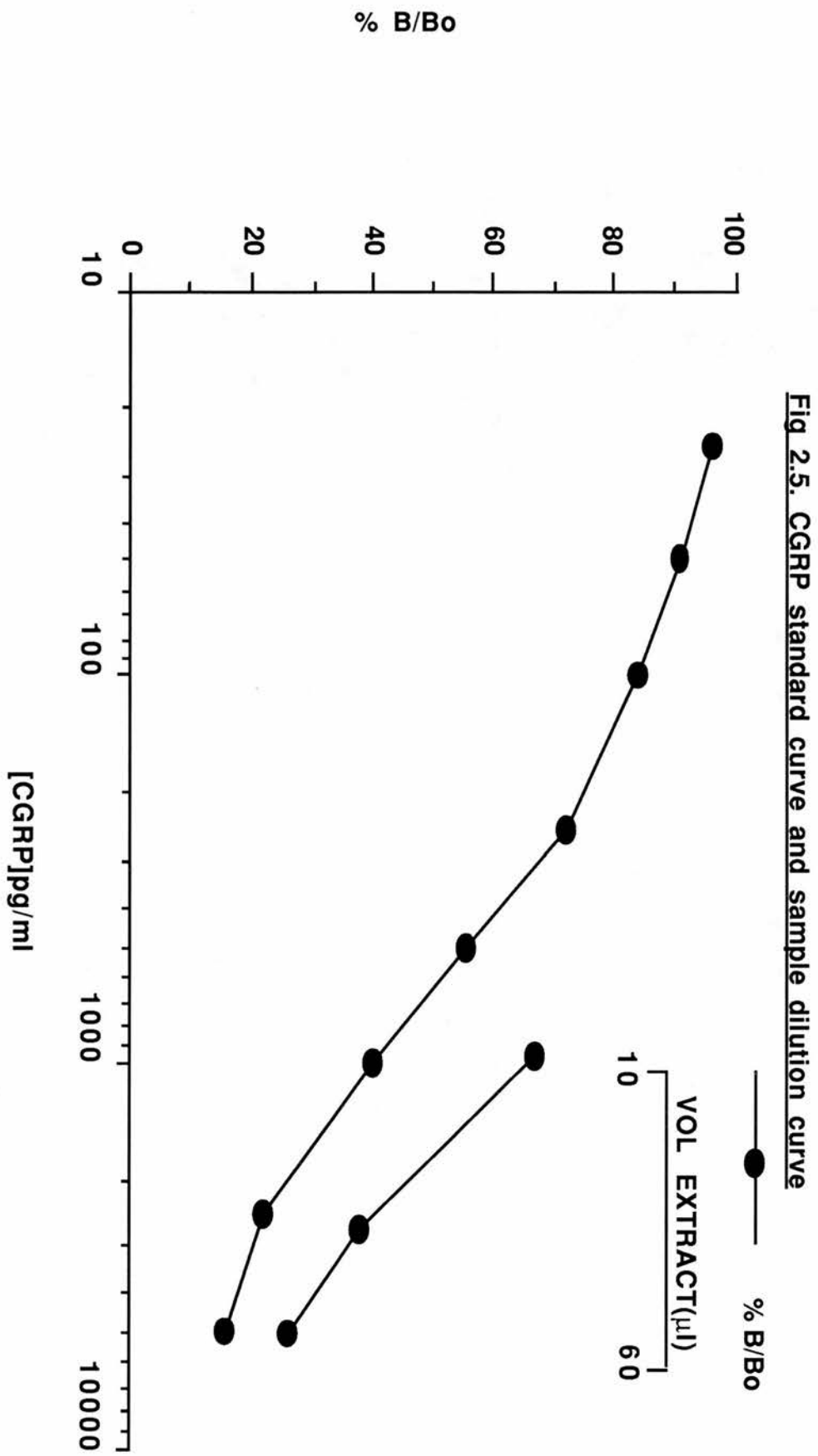
2.3.4.2 Standard Curve

A stock solution of rat CGRP (Peninsula) in 0.1M acetic acid (200µg/ml) was prepared and stored at -40°C. From this stock, aliquots were dried down and standards prepared in duplicate, of 25, 50, 100, 250, 500, 1000, 2500 and 5000pg/ml in 200µl assay buffer. A standard curve was computed by the on-line computer, using a best fit linear regression programme and a log-logit transformation. Unknowns were compared to the standard curve by the computer and reported in pg/ml (see Fig. 2.5).

2.3.4.3 Primary Antibody

A commercial antiserum raised in rabbit against human CGRP was purchased (Amersham International, UK) as a lyophilised powder and stored at -40°C until required. Prior to use, the powder was resuspended in

Figure 2.5 Typical standard dilution curve of CGRP. The dilution curve of CGRP content of homogenised *drg* is also shown



2ml assay buffer and further diluted to $1/10$ th stock in assay buffer. 100 μ l was added to each assay tube.

2.3.4.4 Labelled Antigen

Labelled human CGRP (2-[125 I] iodohistidyl 10 CGRP of specific activity 74 TBq/mmol) was purchased from Amersham International in a 10 μ Ci lyophilisate. This was reconstituted in 100 μ l distilled water and diluted in assay buffer such that 100 μ l contained 5000cpm. 100 μ l was added to each tube.

2.3.4.5 Second Antibody

Separation of bound label from unbound was facilitated by use of ARGG (1:20 dilution) in assay buffer; 300 μ l was added to each tube. 300 μ l of NRS (1:800 dilution) was also added.

2.3.4.6 Quality Control

For comparison between assays and monitoring of intra-assay consistency pools were placed within the assay. Pools consisted of aliquots of a stock solution of rat *drg* homogenised in 2M acetic acid and contained 670pg/ml and 7400pg/ml, respectively. Interassay CoV was < 20% and intra-assay CoV was < 10%.

2.3.4.7 Cross-Reactivity

The antiserum was raised against human CGRP and was reported to cross-react only 30% with the rat sequence. At the highest concentrations tested, the assay did not cross-react with SP (< 0.01%) or SS-14 (< 0.01%).

2.3.4.8 Assay Procedure

Day 1:

Samples in acetic acid were dried down as for the SS assay and resuspended in assay buffer. 200 μ l aliquots of sample or standard were added to 100 μ l of antiserum. The tubes were mixed and incubated overnight at 4°C.

Day 2:

100 μ l of labelled antigen in assay buffer was added, the solutions mixed, and incubated overnight at 4°C.

Day 3:

300 μ l ARGG was added, followed by 300 μ l NRS. The tubes were mixed and incubated overnight at 4°C.

Day 4:

All tubes, except the total counts, were centrifuged at 1,720 x g for 30 mins at 4°C and the supernatants aspirated to leave the precipitate. The radioactivity contained in the precipitates was determined by counting in a gamma counter for 3 min.

2.3.5 Radioimmunoassay for ACTH

2.3.5.1 Buffers

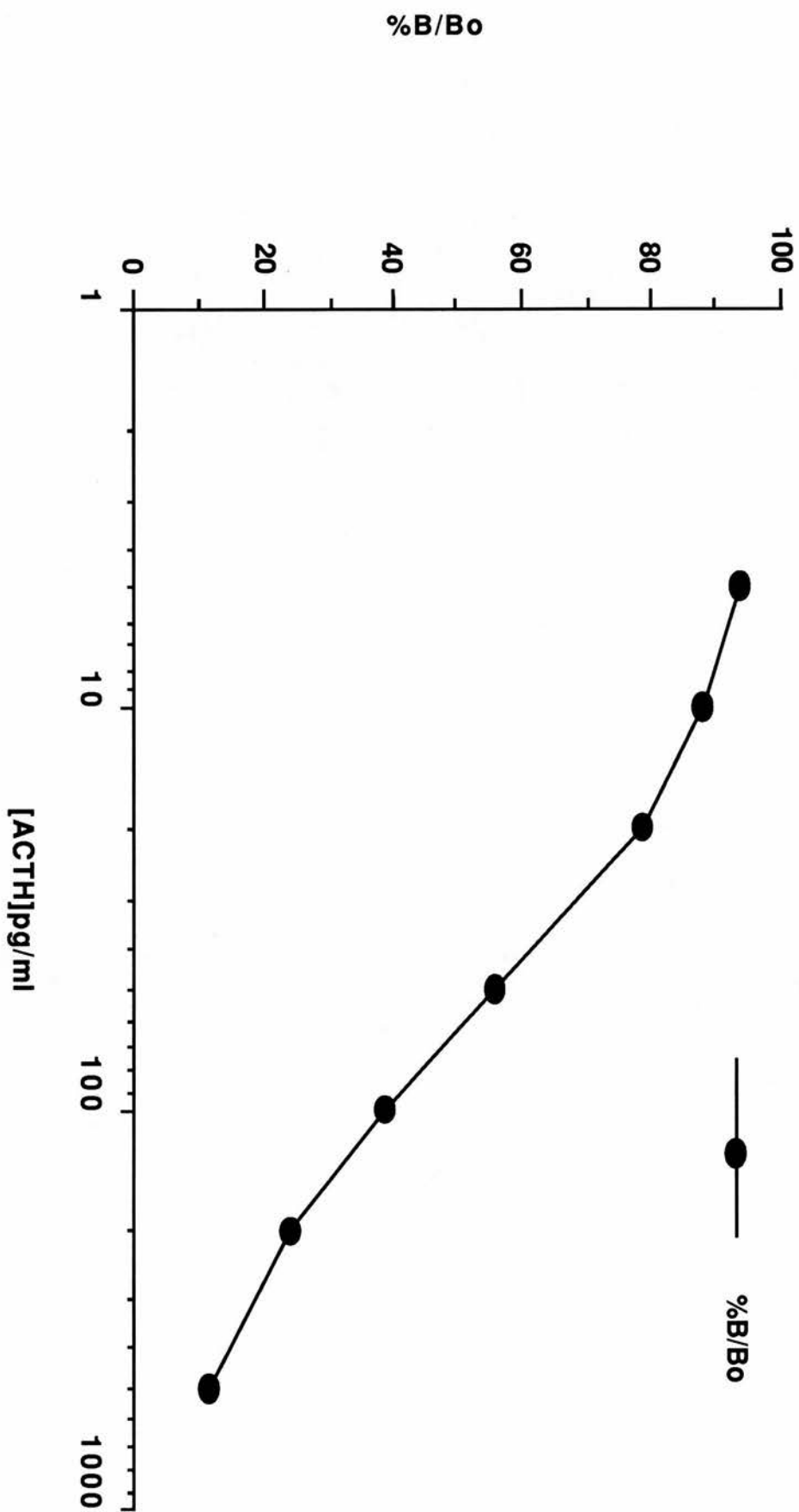
Stock buffer for ACTH assay consisted of 0.05M Na₂HPO₄, 13mM Na₂ EDTA, 0.02% w/v NaN₃ and 200KIU/ml of trasylol, pH 7.4-7.6

2.3.5.2 Standard Curve

A stock solution of human ACTH (Peninsula, 500 μ g/ml) in 0.1M acetic acid was prepared and stored at -40°C. Aliquots of this stock were evaporated to dryness and resuspended in stock buffer containing 3.5% BSA. A standard curve was constructed from duplicates containing 5, 10, 20, 50, 100, 200, and 500pg/ml ACTH. The curve was computed by an on-

Figure 2.6 Typical standard dilution curve for radioimmunoassay of ACTH

Fig 2.6. ACTH RIA standard curve



line computer using a best fitting linear regression programme and a log-logit transformation (see Fig. 2.6).

2.3.5.3 Primary Antibody

The antiserum, raised against human ACTH₁₋₂₄, was purchased as a lyophilised powder (IgG Corporation, USA), reconstituted in 1ml of stock buffer containing 1% v/v NRS (SAPU) and stored at -40°C. Before use, this stock was further diluted in stock buffer and used at a final dilution of 1:6300. The antiserum cross-reacted 100% with rat ACTH.

2.3.5.4 Labelled Antigen

Iodinated human ACTH₁₋₃₉ (3-[¹²⁵I]iodotyrosyl²⁵) was purchased from Amersham International (UK) with specific activity 74TBq/mmol, and diluted in stock buffer containing 0.1% Triton-X-100 such that 100μl contained 10000cpm.

2.3.5.5 Secondary Antibody

The bound labelled antigen was separated from the unbound by precipitation with ARGG. 100μl of a 1:5 dilution in stock buffer containing 0.1% Triton-X-100 was added to all tubes except total counts.

2.3.5.6 Quality Control

Pools of rat plasma samples were added to the beginning and end of each assay for comparison between assays and as a measure of the consistency of conditions within the assay. Pools consisted of previously assayed rat plasma samples and contained 80pg/ml and 660pg/ml ACTH respectively.

2.3.5.7 Assay Procedure

Day 1:

Plasma samples were aliquoted and made up to 100 μ l with stock buffer containing 3.5% w/v BSA. 100 μ l antiserum was added and the tubes incubated overnight at room temperature.

Day 2 (a.m.):

100 μ l labelled antigen in stock buffer containing 0.1% v/v Triton-X-100 was added and incubated for 7 hours at room temperature.

Day 2 (p.m.):

100 μ l ARGG containing 0.1% v/v Triton-X-100 was added.

Day 3:

1.6ml of stock buffer was added to each tube (except total counts) and the tubes centrifuged at 1,720 x g for 30 min at 4°C. The supernatant was aspirated and the precipitate counted in a gamma counter.

2.3.6 Radioimmunoassay for corticosterone

2.3.6.1 Assay Buffer

The standard assay buffer contained 0.1M disodium citrate: 0.2% gelatin w/v at pH 4.0. This was produced using the following protocol. 21.0g of citric acid was dissolved in 200ml 1M NaOH and diluted to 1l with distilled water. To 200mls of this solution gelatin (2g) and thimerosal (0.2g) was added and dissolved with heating. Once dissolved, this was added to the remainder of the citric acid solution and was called solution A. 558mls of solution A was then added to 442mls of 0.1M HCl to give the final buffer at pH 4.0.

2.3.6.2 Standard Curve

Standards containing 0, 500, 1000, 2500, 5000, 10000, 20000 and 40000pg/ml corticosterone were prepared from a solution of corticosterone

in ethanol, evaporated to dryness and reconstituted in charcoal-stripped plasma. They were mixed and allowed to stand at room temperature for 30 min, and 5 μ l aliquots taken in duplicate. The stock solution and prepared standards were stored at -40°C. The standard curve was constructed by an on-line computer using a best fitting linear regression programme and a log-logit transformation. Unknowns were compared automatically by the computer to the standard curve (see Fig. 2.7).

2.3.6.3 Primary Antibody

The antiserum, raised in rabbit against Corticosterone-21-hemi-succinate conjugated to BSA was a kind gift of A. Watts. The antiserum was diluted 1:200 in assay buffer and 500 μ l added.

2.3.6.4 Labelled Antigen

[1,2,6,7-³H] corticosterone with specific activity 2.78 TBq/mol was purchased from Amersham International (UK) and diluted in assay buffer such that 100 μ l contained 10000cpm. 100 μ l was added to each tube.

2.3.6.5 Second Antibody

The bound labelled antigen was precipitated using ARGG. 500 μ l of assay buffer containing 1:100 dilution of ARGG and 1:1000 dilution of NRS was added and incubated overnight at 4°C.

2.3.6.6 Preparation of Samples for Counting

After incubation for at least 16h, the assay tubes were centrifuged at 1720g for 45 min and the supernatant aspirated, leaving the pellet. The pellet was reconstituted in 1ml of 1mM HCl, the solution transferred to counting vials and allowed to stand for 4h. Scintillant was added to each

Figure 2.7 Typical standard dilution curve for radioimmunoassay of corticosterone

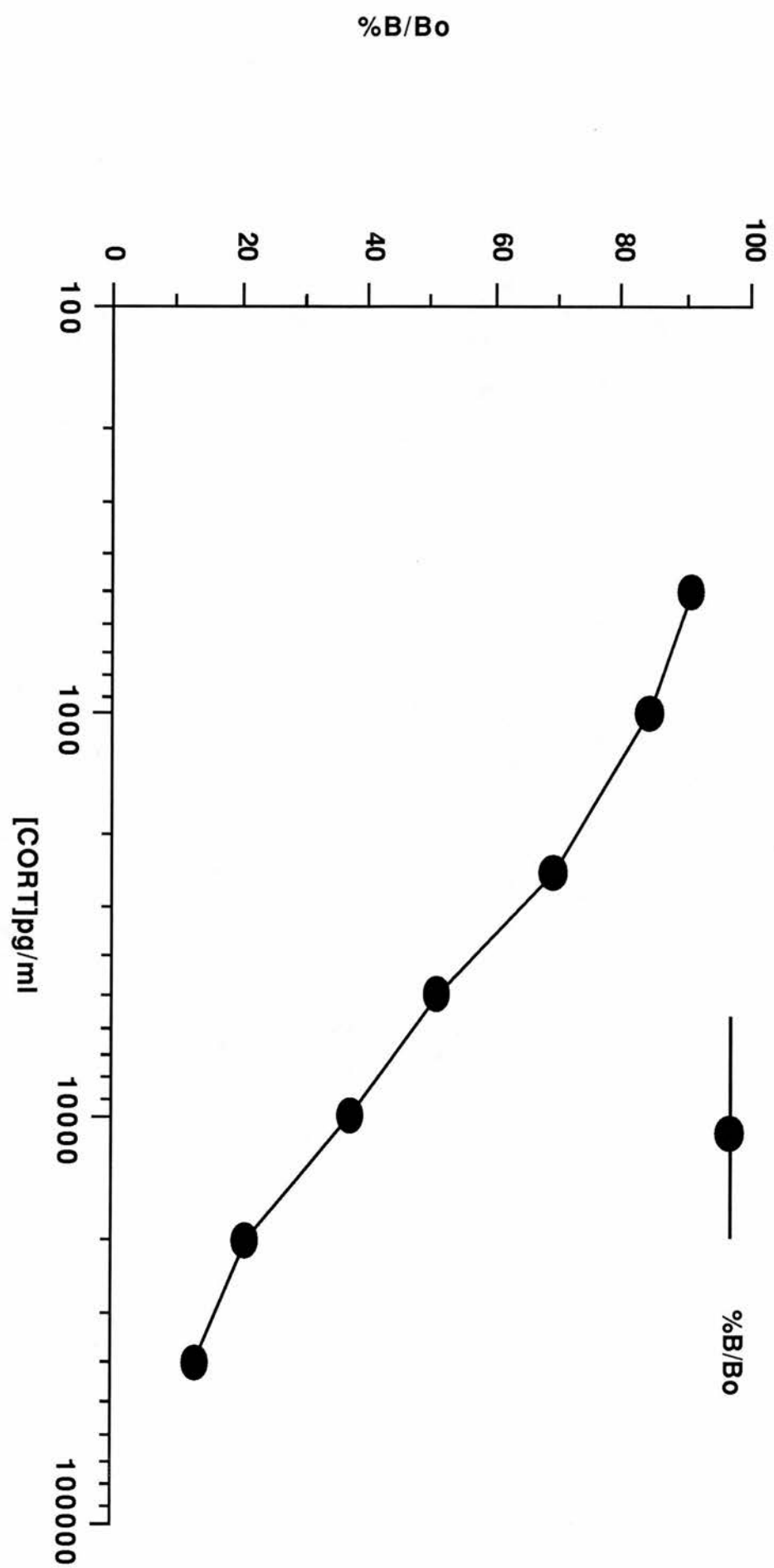


Fig 2.7. Corticosterone RIA standard curve.

vial (4.5mls Aquasafe, Packard) and the radioactivity determined in a β counter by counting for 10 mins.

2.3.6.7 Quality Control

Rat plasma sample pools containing 50ng/ml and 250ng/ml B were added to the start and end of each assay.

2.3.6.8 Assay Procedure

Day 1:

10000cpm of [1,2,6,7- ^3H] corticosterone in 100 μl assay buffer, and 500 μl of 1:200 dilution in assay buffer of antiserum was added to 5 μl plasma, mixed and incubated overnight at 4°C.

Day 2:

500 μl of assay buffer containing ARGG and NRS was added, the tubes mixed and incubated overnight at 4°C.

Day 3:

Tubes were centrifuged at 1720g at 4°C for 45 min and the supernatant aspirated. The pellet was reconstituted in 1ml of 1mM HCl and transferred to a counting vial. After standing for 4h, 4.5ml aquasafe scintillant (Packard) was added and tubes were counted for 10 min in a β counter.

2.3.7 Radioimmunoassay for testosterone

2.3.7.1 Buffers

The standard assay buffer consisted of 0.05M Na_2HPO_4 , 0.05M KH_2PO_4 , 0.14M NaCl, 0.05% v/v NaN_3 , 0.1% w/v gelatin, pH 7.4.

2.3.7.2 Standard Curve

Aliquots of a stock solution of testosterone (100µg/ml in ethanol, Sigma, UK) were evaporated to dryness and reconstituted in assay buffer.

Standards containing 50, 100, 200, 500, 1000, 2000 and 5000pg/ml testosterone in 100µl assay buffer were prepared in duplicate. The standard curve was constructed by an on-line computer using a best fit linear regression programme and a log-logit transformation. Unknowns were compared with the standard curve automatically by the computer (see Fig. 2.8).

2.3.7.3 Primary Antibody

Antiserum raised in rabbit against Testosterone 19-carboxymethyl ether conjugated to BSA was purchased from Radioimmunoassay Systems Laboratories Inc. (USA) and used at a dilution of 1:800,000 in assay buffer.

2.3.7.4 Labelled Antigen

[1,2,6,7-³H] testosterone (Amersham, International UK) with specific activity 3.63 TBq/mmol was used and diluted in assay buffer. 100µl, containing 5000cpm was added to each assay tube.

2.3.7.5 Second Antibody

Bound labelled antigen was separated from unbound by precipitation with ARGG. 500µl of a solution containing ARGG (1:10 dilution) and NRS (1:100 dilution) in assay buffer was used.

The precipitate was pelleted by centrifugation at 2370 x g for 30 min and the supernatant aspirated to leave the pellet. The pellet was reconstituted in 1ml of 1mM HCl transferred to a counting vial and left to stand for 30 min. To this, 4.5ml of aquasafe scintillant (Packard) was added, and samples counted for 5 min in a β counter.

Figure 2.8 Typical standard dilution curve of the radioimmunoassay for testosterone

%B/Bo

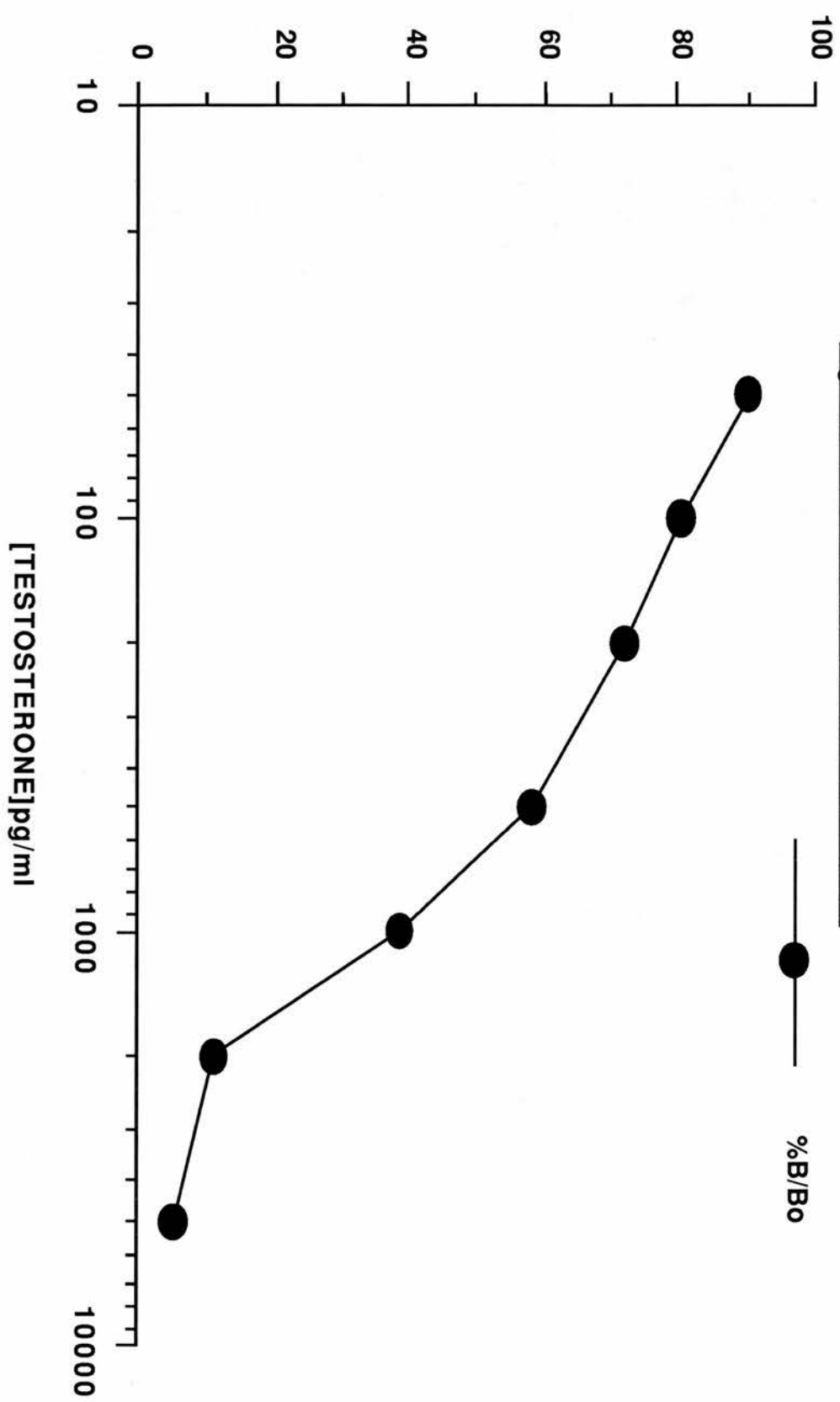


Fig 2.8. Testosterone RIA standard curve

2.3.7.6 Quality Control

Rat plasma sample pools containing 0.8ng/ml, 1.8ng/ml and 6ng/ml testosterone were placed at the beginning and end of the assay.

2.3.7.7 Assay Procedure

Day 1:

20µl of plasma samples were extracted twice in 1ml of diethyl ether. The aqueous phase was evaporated to dryness and reconstituted in 100µl assay buffer. 500µl of a 1:800000 dilution of antiserum in assay buffer and 5000cpm of [1,2,6,7-³H] testosterone in 100µl assay buffer was added, mixed and incubated overnight at 4°C.

Day 2:

500µl of assay buffer containing ARGG and NRS was added, the tubes vortexed and incubated at 4°C for 16h.

Day 3:

The assay tubes were centrifuged at 1720g for 30 min, the supernatant aspirated off and the pellet redissolved in 1ml of 1mM HCl and the solution transferred to scintillation vials. After standing for 30 min, 4.5mls aquasafe scintillant (Packard) were added and the vials counted in a β counter for 5 min.

CHAPTER 3

THE EFFECT OF ADRENALECTOMY AND CORTICOSTEROIDS ON SP, SS
AND CGRP CONTENT OF *DRG* OF RAT

3.1 INTRODUCTION

Adrenal steroids are synthesised from cholesterol in the *zona reticularis* of the adrenal gland. In the rat corticosterone (B) forms the major component and is transported to target tissues in the blood bound to transcortin. Corticosteroids bind to one of two types of intracellular receptor: (i) the Type I receptor ($K_D \sim 0.5\text{nM}$ for B) which binds aldosterone and B with equal affinity but both with much greater affinity than dexamethasone (DEX), and (ii) the Type II receptor ($K_D \sim 2.5\text{-}5\text{nM}$ for B) which binds DEX with greater affinity than aldosterone or B (Reul & de Kloet, 1985). However, circulating levels of B are much greater than aldosterone, yet binding studies using $[^3\text{H}]\text{-B}$ and $[^3\text{H}]\text{-aldosterone}$ indicate that in some tissues (e.g., kidney, parotid gland and colon), the physiological ligand for type I receptors is aldosterone (Sheppard & Funder, 1982). Given the equal affinity of type I receptors for B and aldosterone in the presence of excess B, some additional mechanism must operate to allow selective mineralocorticoid action. There is considerable evidence to suggest that in mineralocorticoid target tissues the presence of the enzyme 11- β hydroxysteroid dehydrogenase serves to limit the access of B to type I receptors (Edwards et al., 1988; Funder et al., 1988). The 11- β steroid metabolite produced by the enzyme has been shown to have considerably lower affinity for both Type I and Type II receptors (Funder et al., 1988) and inhibition of enzyme activity enhances the *in vivo* uptake of B in the rat kidney (Funder et al., 1988; Edwards et al., 1988). Using *in situ* hybridisation histochemistry, in collaboration with Dr. J. Seckl, I have previously demonstrated the presence of mRNA for both types of receptor in *drg* (Seckl et al., unpublished observations).

Plasma corticosteroid concentration is regulated in a negative feedback loop involving adrenocorticotrophin (ACTH), corticotrophin releasing factor (CRF) and arginine vasopressin (AVP). Stimuli promoting

the secretion of corticosterone elicit release of CRF from the hypothalamus which, in turn, mediates the secretion of ACTH from the anterior pituitary gland. AVP potentiates the release of ACTH by CRF. ACTH stimulates synthesis and secretion of corticosteroid by the adrenal cortex, and this then acts to inhibit the secretion of ACTH (see Williams & Foster, 1985).

Following adrenalectomy (ADX) both AVP peptide content and mRNA in the paraventricular nucleus (PVN) are increased, and in the suprachiasmatic nucleus (SCN) peptide and message content are decreased (see McEwen et al., 1986). In the parvocellular population of the PVN, AVP is colocalised with CRF peptide which is also increased following ADX. CRF is also colocalised with enkephalin and neurotensin in the PVN, but the immunostaining for these peptides does not change following ADX. DEX reverses the effects of ADX on CRF in the PVN of long term adrenalectomised rats, but mineralocorticoids are ineffective. CRF receptor numbers also decrease in the anterior pituitary (AP) following ADX, but are unchanged in the cortex and limbic system (Wynn et al., 1984; Sawchenko et al., 1984).

In the adrenal cortex, ACTH binds to specific, high affinity, membrane-bound receptors to increase intracellular c-AMP and activate a number of c-AMP-dependent protein kinases. Following binding of ACTH to its receptor in the adrenal cortex, a rapid increase in the biosynthesis of pregnenolone (an intermediate in the biosynthesis of B) from cholesterol precursor occurs, which subsequently leads to increased biosynthesis of B. In addition to these acute effects of ACTH, chronic treatment of adrenal cells with ACTH leads to increased synthesis of steroidogenic enzymes which also enhances corticosteroid biosynthesis (see Simpson & Waterman, 1983).

ACTH is a product of the proopiomelanocortin (POMC) gene which in the AP also encodes β -endorphin; in the intermediate lobe, tissue specific

splicing of the primary transcript produces mRNA encoding α melanocyte stimulating hormone, corticotropin-like intermediate lobe peptide, γ -lipotropin and β -endorphin. Following bilateral ADX, there is a rapid and substantial rise in plasma ACTH, which is accompanied some 6-8 hours later by stimulation of transcription of POMC mRNA in cells of the AP. This lag in stimulation results in an initial depletion of pituitary ACTH peptide content, but subsequently, some 10 days later a new steady state is achieved in which pituitary ACTH is elevated 3-5 fold greater than in sham-operated animals. In addition, the percentage of AP cells immunoreactive for ACTH doubles, pituitary POMC mRNA levels are increased 2-3 fold and plasma ACTH levels are considerably increased following chronic bilateral ADX (Dallman et al., 1987).

Glucocorticoids have been found to regulate other neuropeptides. Thus, immunostaining for angiotensinogen, the precursor of angiotensin I and II, found in the preoptic area (POA), AP, periaqueductal grey matter and area postrema is decreased following ADX (Wallis & Printz, 1980). Following ADX and colchicine treatment of rats, angiotensin II immunoreactivity is enhanced in the same population of parvocellular neurons that contain CRF (Lind et al., 1984). In the hippocampus, VIP immunoreactivity is decreased following ADX; this effect is reversed by DEX and B (Rotszjetnet et al., 1980). Finally, in rat striatum the expression of preproenkephalin mRNA is decreased following ADX (Chao & McEwen, 1990) and PPT mRNA expression is increased (Chao & McEwen, 1991).

Regulation of SS and CGRP by glucocorticoids has been investigated *in vitro*, in a variety of cell lines. In the rat 44-2C cell line (derived from thyroid C cells) SS content is elevated but CGRP specific mRNA is significantly reduced by DEX (Zeytin & Delellis 1987; Zeytin et al., 1987). In contrast, administration of DEX potently inhibits SS production in TT cells (human medullary thyroid carcinoma cells; Cote et al., 1986), but increases

production of CGRP-specific mRNA (Cote & Gagel, 1986). In the CNS, ADX does not alter SS content of the hypothalamus, cortex, striatum or hippocampus, however SS binding is decreased in hypothalamus, hippocampus and striatum, but not cerebral cortex following ADX (Rodriguez et al., 1988).

In the introduction to this thesis, I have discussed neuropeptides in the *drg* and their putative role in the transmission of pain and neurogenic inflammation, processes which can both be influenced by corticosteroids. It is not known if the analgesic and anti-inflammatory actions of glucocorticoids involve regulation of SP, SS or CGRP in the *drg*, however, corticosteroids have been found to regulate axonal transport of SP and SS in the sensory vagus nerve (MacLean, 1987). As a measure of neuropeptide transport the vagus nerve of male rats was ligated unilaterally and 24 hours later the neuropeptide content of 3mm sections of nerve on either side of the ligation determined by RIA. It was found that daily administration of ACTH for 14 days significantly reduced SP accumulation in the nerve proximal to the site of ligation compared to untreated controls. No difference in SP content of the unligated nerve between ACTH treated and untreated animals was found. However, administration of B over the same time period reduced the accumulation of SP proximal to the ligation. In contrast, ADX increased both SP transport in the ligated nerve and SP content of unligated nerve by approximately 20%. SS transport was also regulated by corticosteroids; following administration of B to supraphysiological levels over two weeks axonal transport of SS increased, but administration of supraphysiological amounts of ACTH or ADX was without effect on SS transport.

A direct relationship between SP axonal transport and SP synthesis in the vagus nerve has been demonstrated previously using studies of ligation and ^{35}S methionine incorporation into SP, subsequently identified by HPLC and immunoprecipitation (MacLean, 1987a; MacLean & Lewis,

1984). Consequently, the observation of increased SP axonal transport following ADX probably reflects an increase in SP synthesis in the ganglion. In addition, whilst SS axonal transport in the vagus is unaltered following ADX, supraphysiological levels of B increases axonal transport of SS and thus, there would appear to be a reciprocal regulation of SS and SP by corticosteroids in the vagus.

Given the presence of mRNA for both types of corticosteroid receptors in the *drg*, and the role of these steroids in the regulation of neuropeptide gene expression, biosynthesis and transport, I have examined the role of corticosteroids in the regulation of neuropeptide content of the *drg*.

3.2 METHODS

Ham Wistar rats (male, 200g) were adrenalectomised or sham operated under halothane anaesthesia using the two step method described previously (Chapter 2). Animals were allowed to recover and five, ten or thirty days after ADX, were killed by rapid decapitation, and trunk blood samples collected on ice for determination of plasma B and ACTH by RIA (see Chapter 2). *Drg* from cervical level C4, C5, C6 and C7 were collected and SP, SS or CGRP content determined by RIA as described (Chapter 2). Experiments investigating the time course of any effects of ADX on neuropeptide content of cervical *drg*, and the role of glucocorticoids and mineralocorticoids in these actions were performed.

To examine the time course, animals were adrenalectomised or sham operated and implanted subcutaneously with 200mg pellets of wax or containing 50% B (Sigma, UK)/cholesterol (Sigma, UK); pellets were inserted under halothane anaesthesia subdermally in the scruff of the neck, and replaced after fifteen days. Five, ten and thirty days after surgery, animals were killed by rapid decapitation, *drg* dissected and SP and SS content determined. Contents were expressed in pg/ganglion and given as

mean \pm S.E.M. Results were analysed by ANOVA followed by Duncan's Multiple Range Test for post hoc comparison.

Having established the optimum period over which to examine the effects of ADX on neuropeptides, the role of mineralocorticoids and glucocorticoids were examined using B (Sigma, UK), DEX (Sigma, UK) and the specific antiglucocorticoid RU38486 (Roussel, France). Animals were adrenalectomised or sham operated, and received daily s.c. injection of DEX (0.2mg/kg), B (2.5mg/kg) or RU38486 (10mg/kg) in sesame oil vehicle. Sham operated controls and one group of adrenalectomised animals received daily s.c. injection of vehicle. Ten days after surgery, animals were killed and plasma samples and *drg* collected as previously. Mean SP, SS and CGRP contents (pg/ganglion) of C4, C5, C6 and C7 *drg*, were determined by RIA, results pooled, and expressed as a percentage of that of sham operated controls. Results were analysed as above.

3.3 RESULTS

3.3.1 Time Course

The plasma B and ACTH concentrations following sham operation, ADX or pellet replacement are given in Table 3.1. Plasma B of animals following ADX was below the limit of sensitivity of the assay ($< 2.9\text{ng/ml}$) and pellet replacement increased this to approximately one quarter that of sham-operated controls. ADX elevated plasma ACTH compared with sham-operated animals and pellet replacement partially reversed this increase in plasma ACTH.

Mean SS content of cervical *drg* was significantly decreased five, ten and thirty days following ADX compared with sham operated controls (Fig. 3.1a). B administration in the form of subcutaneous pellet implant partially reversed the effects of ADX over the thirty day period. SS content was not evenly distributed over the four ganglia studied; SS content (Fig 3.2a) was

Table 3.1 Plasma B (ng/ml) and ACTH (pg/ml) of animals following sham operation, ADX or ADX with pellet replacement

TABLE 3.1

	SHAM	ADX	B-REP
Plasma Corticosterone (ng/ml)	227.1 ± 26.8	< 2.9	57.4 ± 9.8
Plasma ACTH (pg/ml)	230.5 ± 104.2	1114.5 ± 125.7	844.7 ± 339.5

Figure 3.1 Mean SS (a) and SP (b) content (pg/drg) of *drg* from C4 to C7 following sham operation, ADX for five, ten or thirty days (ADX 5, ADX 10, ADX 30) or pellet replacement of B following ADX (B-REP)

* $p < 0.05$, ** $p < 0.01$, c.f. sham operated control (ANOVA, post-hoc Duncan's Multiple Range test)

Fig 3.1a.

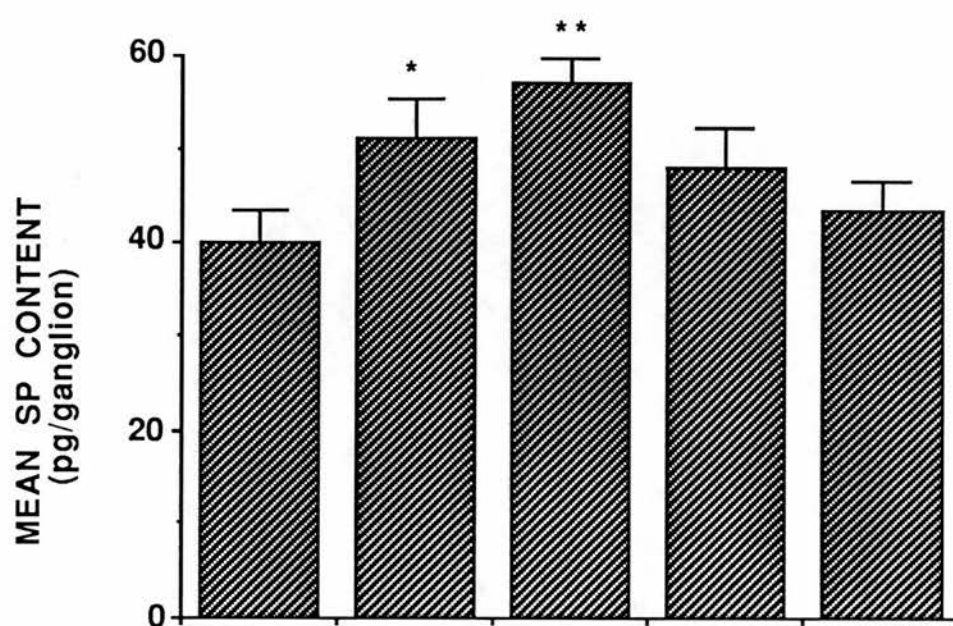


Fig 3.1b.

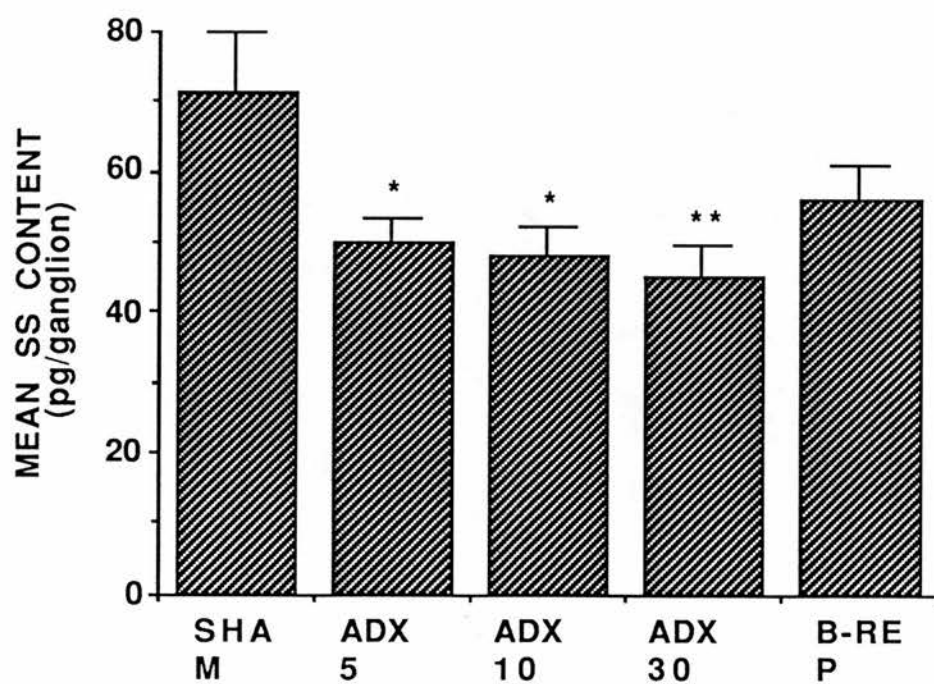


Figure 3.2 Distribution of SP (a) and SS (b) content (pg/*drg*) in *drg* from C4 to C7, and the SP content following sham operation, ADX for five, ten or thirty days (ADX 5, ADX 10, ADX 30) or pellet replacement of B (B-REP)

Fig 3.2a.

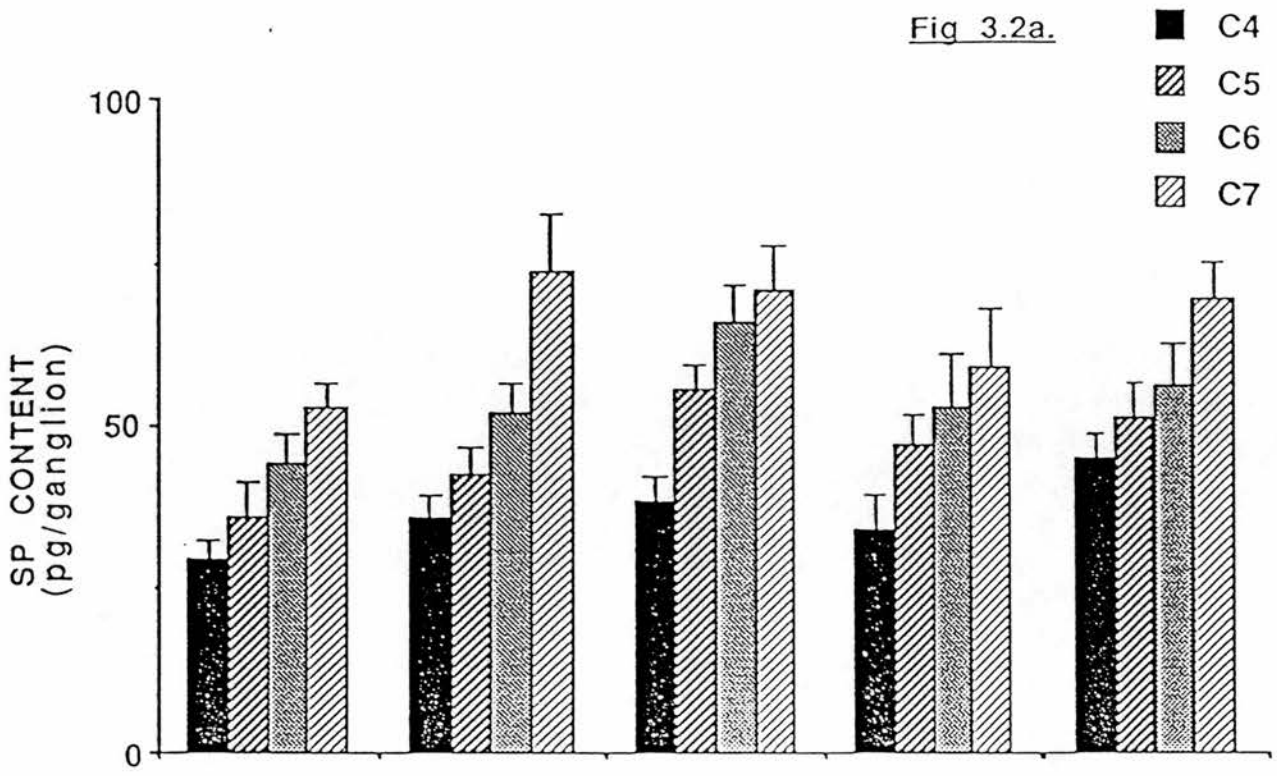
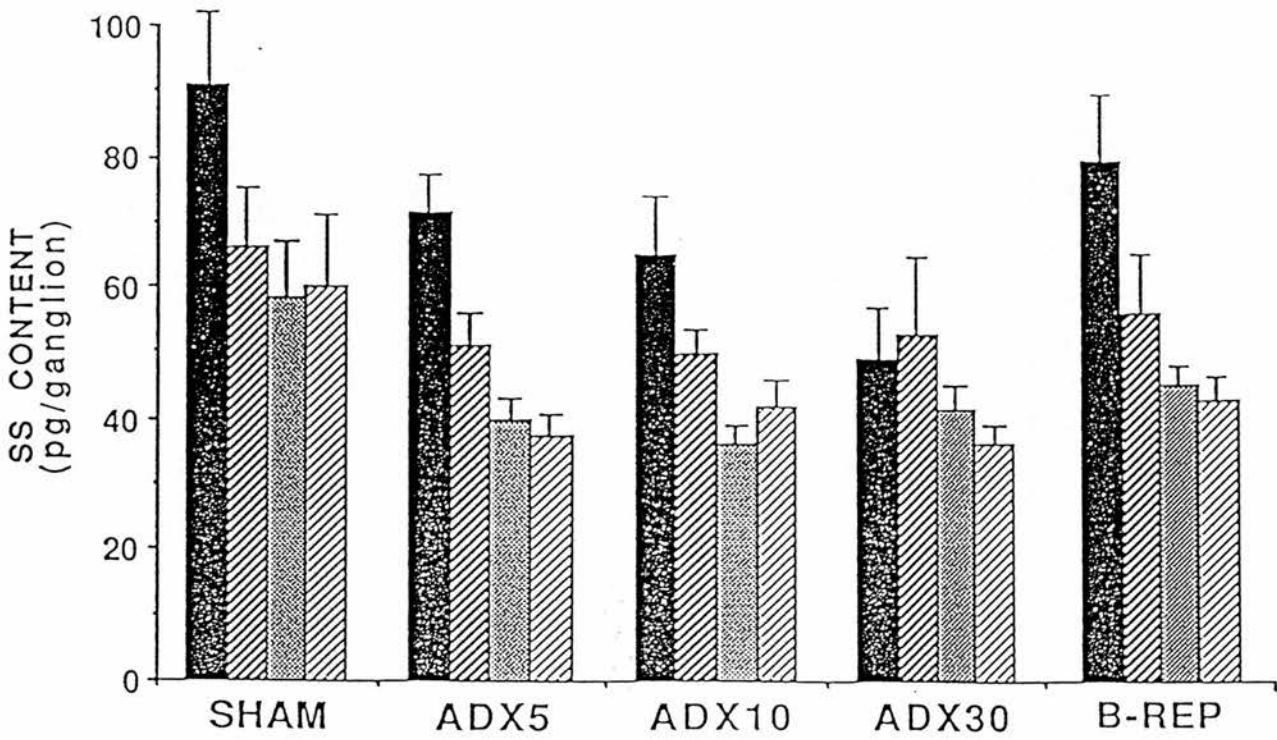


Fig 3.2b.



greatest in C4 compared with C5, C6 or C7. Thirty days after ADX the differential distribution of SS was no longer present: B replacement over the same period restored the distribution to that of controls.

SP content (Fig. 3.1b) was significantly increased five and ten days following ADX compared to sham operated animals. However, this increase in SP content was not sustained, and thirty days after ADX, SP content was no longer significantly different from that of sham operated controls. SP content of *drg* from adrenalectomised animals receiving B was not significantly different from that of sham operated controls over the 30 day period. SP content (Fig. 3.2b) was least in C4 and C5 and greatest in C7. This distribution of SP was maintained five, ten and thirty days following ADX.

3.3.2 Replacement Studies

As was expected, plasma B (Table 3.2) of adrenalectomised animals was greatly decreased (below the limit of sensitivity of the assay, < 2.9ng/ml) and plasma ACTH was increased compared with sham-operated animals. DEX replacement did not alter the effects of ADX on plasma B, but suppressed plasma ACTH below that of controls. Daily s.c. injection of B only slightly increased plasma B above that of adrenalectomised animals receiving no treatment and did not reverse the increase of plasma ACTH. Treatment of intact animals with RU38486 did not significantly alter plasma B but suppressed plasma ACTH below that of controls.

As in the previous experiment, 10 days following ADX, SP and CGRP content (Fig. 3.3a,c) of cervical *drg* was significantly increased compared to sham operated controls, SS content (Fig. 3.3b) was decreased. In contrast, SP and SS content of *drg* from adrenalectomised animals treated with DEX was not significantly different from controls. Similarly, DEX treatment

Table 3.2 Plasma B (ng/ml) and ACTH (pg/ml) of animals following ADX, ADX and administration of DEX or B or treatment of intact animals with RU38486

TABLE 3.2

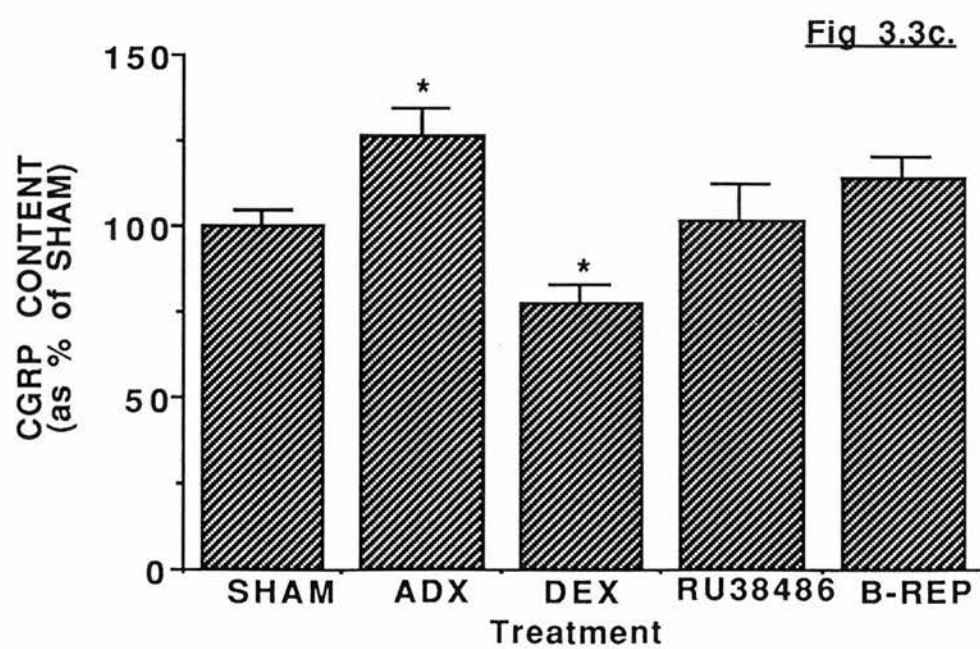
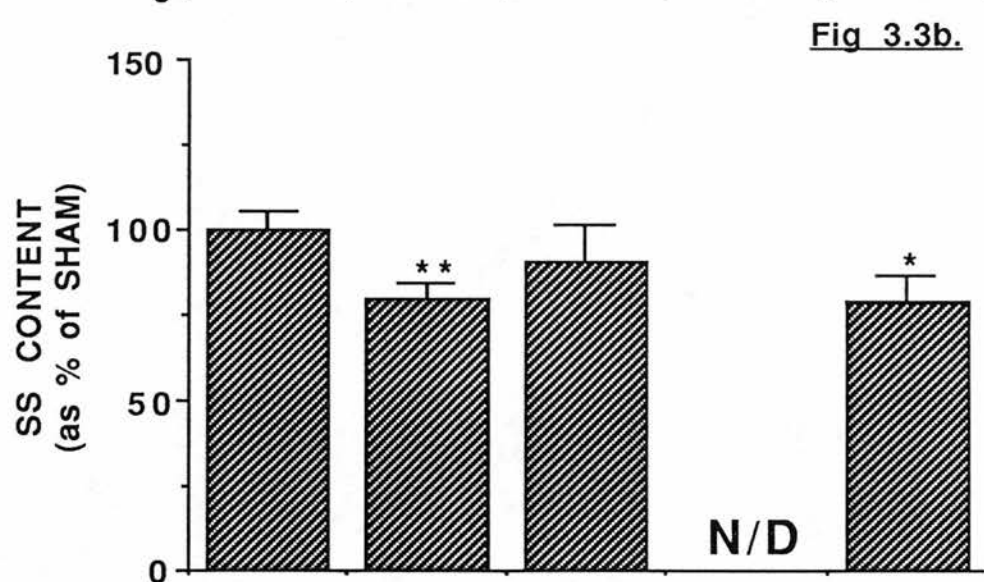
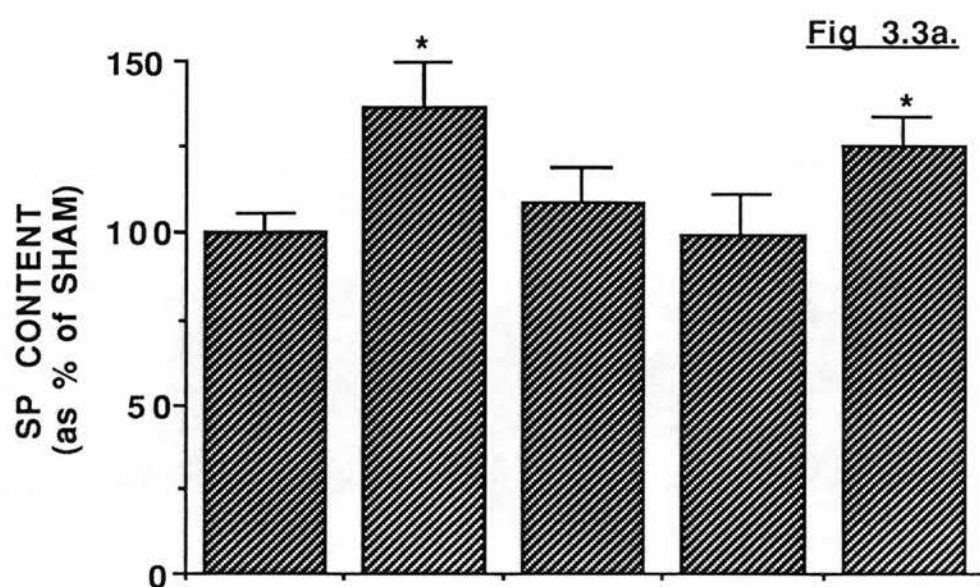
	SHAM	ADX	B-REP	DEX	RU38486
Plasma Corticosterone (ng/ml)	382.3 ± 65.8	< 2.9	9.5 ± 4.7	< 2.9	313.7 ± 49.4
Plasma ACTH	316.3 ± 75.2	641.4 ± 143.0	675.0 ± 199.6	73.3 ± 12.8	97.9 ± 13.6

Figure 3.3

Mean SP (a), SS (b) and CGRP (c) content of *drg* (% of sham operated controls) following ADX, ADX and daily treatment with DEX or B or treatment of intact animals with RU38486

* $p < 0.05$, ** $p < 0.01$, c.f. sham operated control
(ANOVA, Duncan's Multiple Range Test)

N/D not determined



reversed the effects of ADX on *drg* CGRP content and further suppressed CGRP content significantly below that of controls.

In contrast to DEX treatment and the results of the previous experiment the method of B replacement used in this experiment did not reverse the effects of ADX on SP content or SS content. B replacement did however reverse the effects of ADX on CGRP content of *drg* but did not suppress CGRP content below that of controls. RU38486, the glucocorticoid antagonist administered to intact animals by daily s.c. injection was without effect on SP or CGRP content of cervical *drg*.

3.4 DISCUSSION

In the experiments described above, I have demonstrated the regulation of neuropeptides in *drg* by glucocorticoids. SS content decreased, but SP and CGRP content increased five and ten days following ADX. Thirty days after ADX, whilst SS content remained suppressed, SP content was no longer significantly different from that of sham operated controls. B replacement by subcutaneous pellet implant reversed the effects of ADX on both SS and SP content, restoring peptide contents to control levels. Similarly, DEX given subcutaneously in oil reversed the effects of ADX on SP and SS content and suppressed CGRP content below that of controls. CGRP content of adrenalectomised animals was restored to control levels following s.c. injection of B, but in contrast to pellet replacement of B, injection did not reverse the effects of ADX on SP or SS content. RU38486 a specific glucocorticoid antagonist was administered by s.c. injection to intact animals but was without effect on neuropeptide content of *drg* by this route of administration. I have also described differential distributions of neuropeptide content of the 4 *drg* (C4-C7); these results have been further investigated and are discussed fully in Chapter 7. The SP gradient did not change following ADX, but the gradient of SS was

abolished 30 days after ADX, and this was restored by replacement of B in the form of s.c. pellet implantation.

As the adrenal gland is the sole site of synthesis of corticosteroids, following ADX endogenous corticosteroid will be completely removed from the animal. The success of ADX was gauged from the absence of B in the plasma and upon inspection, the absence of adrenal fragments in cadavers. Further evidence of the removal of corticosteroids following ADX was indicated by the increase in plasma ACTH which resulted from the removal of the negative feedback of B on ACTH biosynthesis and secretion.

DEX (9 α -fluoro-16 α -methyl-prednisolone) has a long half-life in the body and some specificity for Type II receptors, although at a high dose is active at both Type I and Type II receptors. In my method of administration, daily s.c. injection will maintain pharmacologically active levels of DEX between doses. Thus, Type II receptors will be occupied continuously, and there may be some occupation of Type I receptors. DEX injection reversed the effects of ADX on SP content of cervical *drg*, similarly SS content was restored to sham levels after DEX. CGRP content of cervical *drg* was suppressed significantly below that of sham operated controls. These results suggest that corticosteroid regulation of SS, SP and CGRP is mediated by Type II receptors. That CGRP content was suppressed below that of controls suggests that CGRP was more sensitive to steroid regulation than SP or SS.

In the current study two paradigms were used for B replacement. In the first, a pellet containing 50% B in a 50% cholesterol mixture was inserted subcutaneously, and thus provided a constant moderate dose of B (Dallman et al., 1989). Thus, in this model, Type I, and to a lesser extent, Type II receptors were likely to be occupied throughout the period of the experiment. I have described in the Introduction to this Chapter, the relationship between plasma ACTH and B, which reciprocally regulate each

other in a negative feedback loop. Before considering the plasma B of animals it should be noted that animals were killed in the morning and thus, given the circadian nature of B secretion, plasma B would be expected to be near a minimum. However, plasma B may be elevated as whilst great care was exercised to minimise the stress on animals prior to decapitation, handling and transfer of animals from the animal house to the laboratory prior to sacrifice may have resulted in a stress induced release of B from intact animals. Consequently it may be that the measured plasma B of intact animals was higher at the time of sacrifice than it was for the previous period of the experiment. Thus, whilst the measured plasma B level of pellet replaced animals was approximately one quarter that of sham-operated animals it may be that the model was in fact more effective at replacement of plasma B than is immediately apparent from these figures.

I have found that pellet replacement of B reversed, but not fully, the effects of ADX on SS and SP content of cervical *drg*, and thus this model of replacement appears not to completely mimic the intact animal. The plasma ACTH levels of replaced animals, whilst variable, also indicates that replacement was incomplete; compared with sham-operated controls, the plasma ACTH of replaced animals was elevated (by 3-4 fold), but less than that of ADX animals (5-fold greater than sham).

The second model of replacement of B involved daily s.c. injections of B in oil. In this model, animals will experience a rapid rise in plasma B which will be metabolised between injections, thus given that the injections were administered daily at approximately 4.30 p.m., this model mimics the endogenous circadian rhythm in plasma B. However, as B is rapidly metabolised, the plasma B will approach zero before the second injection is administered. Consequently, when initially injected Type I and Type II receptors will be transiently occupied. However, due to rapid metabolism of B, it is presumed that for the majority of the period plasma B will be at a level

such that only Type I receptors will be occupied, and for some time before the next injection only a proportion of these receptors will bind B. Thus, the animal will only transiently be replaced.

That measured plasma B was extremely low, was to be expected given that animals were killed in the morning some 16 or 20 hours after the last injection of B. Thus, the plasma B, given in Table II, which was just above the limit of sensitivity of the assay was effectively the end point and is of little use in considering the active dose of B achieved in this model. However, this method of B replacement was ineffective in preventing the increase in plasma ACTH following ADX and thus did not effectively mimic the endogenous suppression of B on ACTH biosynthesis and secretion. Furthermore, this method of replacement did not reverse the effects of ADX on SS or SP. However, whilst the effects of ADX on SS or SP were not reversed by daily s.c. injection of B, the CGRP content of *drg* in this treatment group were not significantly different from sham operated controls. These results suggest CGRP might be more sensitive to corticosteroid regulation than SP or SS. The results of the experiments using DEX treatment also suggested CGRP was more sensitive than SP or SS.

RU38486 (11 β -(4-dimethylaminophenyl)-17 β -hydroxy-17 α -propyl-1-vinyl) estra-4,9,dien-3-one; Mifepristone) is a potent antiglucocorticoid with, in addition, antiprogestin activity. It is an antagonist at both the progesterone and glucocorticoid receptors and has 3-fold greater affinity than DEX at the Type II receptor (Agarwal, 1984). In ADX animals, RU38486 has been found to inhibit glucocorticoid induction of tryptophan pyrolase, tyrosine transaminase as well as stimulation of gluconeogenesis (Agarwal et al., 1987). Clinically, the antiprogestin activity of RU38486 allows its use as an abortifacient, and it is also proving useful in the treatment of Cushing's syndrome, but as yet, has been found to possess no agonist activity at the Type II receptor.

In the model of administration used, daily s.c. injection in oil, RU38486 did not significantly alter neuropeptide content compared with sham operated animals. Two conclusions are possible from this experiment: (i) Type II receptors are not important in corticosteroid regulation of neuropeptides in *drg* or, (ii) my route of administration of RU38486 failed to produce pharmacologically useful doses in the animal. The action of DEX to reverse the effects of ADX argues against the assertion that Type II receptors are not involved in the regulation of neuropeptides in the *drg*. Consideration of plasma ACTH levels in animals receiving RU38486 compared with sham operated controls indicates that RU38486 suppressed plasma ACTH. This finding is contrary to the expected outcome of using a glucocorticoid antagonist in intact animals, as this would tend to inhibit glucocorticoid suppression of ACTH synthesis and secretion and consequently lead to an increase in plasma ACTH. From the evidence available it is not clear why in this experiment administration of RU38486 resulted in this apparent anomaly. However, RU38486 is normally administered orally by gavage or in drinking water and it may be that my route of administration was not suitable. I conclude that my method of administration was not appropriate for RU38486.

In the vagus nerve following ADX, axonal transport of SP, but not SS, was increased and, in contrast, following administration of supraphysiological amounts of B, axonal transport of SS, but not SP, was increased (MacLean, 1987). Using studies of ligation and ³⁵S methionine incorporation, a direct relationship between axonal transport of SP and synthesis of SP in the vagus nerve has been demonstrated (MacLean, 1987a; MacLean & Lewis, 1984). It is possible, therefore, that increased transport of SP in the vagus following ADX reflects or is supported by increased synthesis of SP and thus SP in the sensory vagus and *drg* would appear to be similarly regulated by corticosteroids. The reciprocal

regulation by corticosteroids of SP and SS transport in the vagus nerve is also in agreement with my finding that SP and SS content of *drg* changed in opposite directions following ADX.

The rat 44-2C cell line (derived from thyroid cells) has also been used to study glucocorticoid regulation of neuropeptides. Thus, following culture in the presence of DEX, SS content is elevated and mRNA encoding CGRP is significantly reduced (Zeytin & Delellis, 1987; Zeytin et al., 1987). My findings in the *drg*, that DEX reversed the reduction of SS content and increase in CGRP content following ADX are consistent with studies in the 44-2C cell line. In contrast, in the TT cell line (derived from human medullary thyroid carcinoma cells) DEX treatment reduces SS production, indicating glucocorticoid regulation of SS differs in this cell line. In the CNS, SS content of the hypothalamus, cortex, striatum and hippocampus is not changed following ADX, but SS binding is reduced in the hypothalamus, hippocampus and striatum but not cerebral cortex (Rodriguez et al, 1988). SS binding is also reduced on pituitary cells in culture following administration of glucocorticoids (Schonbrun, 1982). In rat striatum, PPT mRNA is regulated by glucocorticoids such that PPT mRNA content is reduced five days following ADX compared with animals receiving B replacement following ADX (Chao & McEwen, 1991).

In the current study RIA was used to examine the neuropeptide content of *drg* following ADX; whilst this technique measures total peptide content it provides no insight into the regulation of neuropeptides within individual cells. The distribution of neuropeptides in the *drg* was described in the introduction to this thesis (Chapter 1) and it is apparent that whilst there is co-localisation of the three neuropeptides under study, discrete populations may also exist (notably of SP and SS). The question therefore arises as to the proportion of the population of peptidergic cells that respond to steroids with a change in neuropeptide content. It may be that the

relatively modest changes in neuropeptide content detected by RIA in this study underlies a far greater change in a subset of the population of peptidergic cells that is masked by a larger population of unresponsive cells. Use of the semi-quantitative technique of immunohistochemistry for the three neuropeptides would allow determination of the specific subpopulation of neurons, perhaps delineated on the basis of cell diameter which might be responding to glucocorticoids. Alternatively it may be that the changes in neuropeptides occurs in only those populations of cells colocalising the peptides under study; the availability of a number of immunohistochemical techniques whereby one can visualise more than one peptide in a single tissue would be of use in resolving these questions.

Whilst I have found a reduction in SS content and an increase in SP and CGRP content following ADX, there is no indication from this study as to the mechanisms responsible for these changes. In the *drg* neuropeptides such as SP, CGRP and SS are synthesised in the cell body and transported in axons or nerve roots centrally or peripherally. Thus, an increase in peptide content might reflect reduced axoplasmic transport of the peptide from the *drg*, and this would be demonstrated using the ligation methods of MacLean described in the introduction to this chapter. Alternatively, increased peptide content might reflect an increase in the synthetic rate of peptide which might be shown by incorporation of radiolabelled amino acids into immunoreactive peptide (Harmar & Keen, 1982). The obverse of the above argument would apply for a reduction in peptide content. Finally, the peptide content could be altered as a consequence of a change in the degradation of peptide in the cell body.

Peptide synthesis can also be regulated by actions of agents on the genome or mRNA. If the availability of mRNA encoding neuropeptides is raised and translation and processing mechanisms are not limiting, the peptide biosynthesis will also be increased and reflected in an increase in

neuropeptide content of the *drg*, all other factors remaining constant. Increases in the availability of specific mRNAs for translation can arise through a number of mechanisms. In the cell body, there is a continual turnover of mRNA and thus a reduction in degradation or increase in synthesis of mRNA will increase availability of mRNA for transcription. It would, therefore, be interesting to examine the content of mRNA encoding SP, SS or CGRP of *drg* following ADX to determine if the effects on neuropeptide content are mediated at the level of mRNA. This experiment might be conducted using of northern blots, nuclease protection assays or in-situ hybridization employing specific cDNA probes for the three neuropeptide mRNAs.

The use of nuclease protection assays might be particularly appropriate, as the technique not only provides information regarding mRNA content (as would be obtained from the Northern Blot) but can also reveal any alterations in the pattern of splicing of the primary transcript. The splicing of the mRNA encoding the three peptides and the other possible products that the mRNA can yield was described in the introduction to this thesis (Chapter 1). The splicing patterns of mRNA are of interest as it has been demonstrated previously in ovine thyroid C-cells that NGF can alter the splicing of the calcitonin/CGRP gene primary transcript to produce CGRP rather than calcitonin, the product in untreated cells. Examination of the mRNA content by in-situ hybridization would also provide information not gained by the use of the other two techniques. In addition to being semi-quantitative, in-situ hybridization would also reveal the population of cells expressing neuropeptide genes that were regulated by ADX.

Whilst I have demonstrated that following ADX, SP and CGRP contents are increased and SS content of *drg* decreased and that these effects are reversed by corticosteroids, the results do not establish the site of action of corticosteroids in the regulation of neuropeptides in the *drg*. It may

be that corticosteroids act at some distant site and influence release of a mediator in the form of a hormone or paracrine factor which subsequently regulates neuropeptides in the *drg*. Alternatively, the mRNA encoding both Type I and Type II receptors has been localised in neurons of the *drg* by *in situ* hybridisation (Seckl et al., unpublished observations) and thus the glucocorticoids may act directly on the *drg*. An experiment to determine if indeed corticosteroids do act directly at the *drg*, (although this does not, by itself, obviate additional secondary actions) would be to examine steroid regulation of neuropeptides in dissociated primary cultures of *drg* in defined medium. This experiment has been performed and is described in Chapter 6.

Neuropeptides in the *drg*, including SP, SS and CGRP have been implicated in the transmission of pain to the dorsal horn and in inflammatory processes (see Chapter 1). Clinically, glucocorticoids are used as anti-inflammatory agents in a variety of conditions including rheumatoid arthritis. It is generally accepted that glucocorticoids influence the immune system and act generally to inhibit the body's natural response to tissue injury (see Chapter 1). It may be, in the light of my results, that in addition they also in part act on the *drg* to regulate availability of neuropeptides in peripheral and central terminals, and thus influence neurogenic inflammatory processes and transmission of nociceptive information. A commonly used model of inflammatory disease is the adjuvant arthritic rat, and in Chapter 5, I have investigated the role of the neuropeptides SP, SS and CGRP in the *drg* in this model. In addition, I have also determined the effects of manipulation of corticosteroids on indices of inflammation and neuropeptides in the *drg*.

In conclusion, I have shown that the neuropeptides SS, SP and CGRP were not evenly distributed in *drg* from the cervical region of the rat. ADX reduced the mean content of SS, but increased SP, and CGRP and this change in the neuropeptides was reversed by pellet replacement of

corticosterone or daily injection of DEX. These results suggest that corticosteroids differentially regulate SS, SP and CGRP content of cervical *drg* in the adult rat, and that this action may be mediated by glucocorticoids, through Type II receptors.

CHAPTER 4

ANDROGENS DO NOT REGULATE SP, SS OR CGRP CONTENT OF *DRG*

4.1 INTRODUCTION

Testosterone (T) is the major steroid produced and secreted by the testes in the normal adult male. T is carried in the bloodstream to target tissues where, being lipid soluble, it crosses the plasma membrane and binds to the androgen receptor, a soluble intracellular protein of some 110-120K_{da} present in the cytosol, (Johnson et al., 1987). Subsequently, the hormone-receptor complex acquires a greater affinity for chromatin and binds to specific areas of the genome to influence gene transcription (Ostrowski et al., 1982; Heynes et al., 1977; Mainwaring et al., 1974). In addition, a number of metabolites of T are biologically active in target tissues. Thus, T can be converted by the intracellular enzyme 5 α reductase, present in target tissues, to its more active congener 5 α dihydrotestosterone (5 α DHT) which also binds to the androgen receptor to influence gene transcription (Bruchovsky & Wilson, 1968; Denef et al., 1973; Denef et al., 1974). Oestradiol is another active metabolite of T formed by the action of aromatase (Ball & Knopper, 1980) and oestrogens, acting again at the level of the genome in a hormone-receptor complex, mimic the differentiating effects of T in some areas of the CNS (Nordeen et al., 1986; Gorski & Wagner, 1965). Finally, in liver and bone marrow, T can be converted to 5 β metabolites which effect androgen actions (see Bardin & Catterall, 1981; Urabe et al., 1979).

Receptors for androgen and oestrogens have been localised to areas of the rat brain using biochemical and autoradiographic techniques (see McEwen, 1980). Androgen receptors are present throughout the CNS, but are concentrated in the hypothalamus, septum, preoptic area and amygdala, and recognise both T and the metabolite 5 α DHT. Whilst oestrogen receptors are found in high concentration in anterior pituitary and to a lesser extent in the basomedial hypothalamus, preoptic area, corticomedial amygdala and mesencephalic gray matter of the rat, a necessary

prerequisite for their mediation of androgen action is the presence of aromatase activity. Aromatase activity is in high concentration in the amygdala, and to a lesser degree, in the preoptic area and hypothalamus, but is absent from the pituitary. This differential distribution of aromatase activity is shown by the fact that following injection of [^3H]-testosterone oestrogen receptors in the amygdala, hypothalamus and preoptic area, but not the pituitary are labelled with [^3H]-oestradiol. Thus, the ability of a tissue to aromatise oestrogen appears to be the limiting factor in oestrogen-mediated effects of T; in contrast 5α reductase activity is present throughout the CNS (see McEwen, 1980).

Sexually dimorphic patterns of sexual behaviour and gonadatrophin secretion in adults results from the presence or absence of androgen (or its metabolites) during the perinatal period (see Beyer & Feder, 1987). In areas of the CNS concerned with the control of reproductive processes including areas of the hypothalamus, medial preoptic area, amygdala, septum and some cells in the brain stem and spinal cord, sex related differences in a variety of morphological parameters such as size of nuclei in neuronal groups, number of neurons, neuronal volume, extent of dendrite elaboration and branching, number of dendritic spines, morphology and localisation of synapses and cell nuclear volume and structure are apparent. In the limbic regions, sex differences in the pattern of connectivity between areas related to reproductive function have also been identified. Perinatal castration of genetic (XY) males demasculinises and feminises those areas of the brain underlying sexual behaviour and feminises the brain-pituitary axis such that female patterns of gonadotrophin secretion are apparent in adulthood. Conversely, injection of androgen into genetic (XX) females masculinises and defeminises these same reproductive processes. Thus, sex steroids, including androgens, have important actions on the brain during ontogeny (see Beyer & Feder, 1987).

Patterns of neuropeptide distribution in certain regions of the CNS and pituitary gland have also been found to be sexually dimorphic. Thus, in the lateral septum and lateral habenula, more fibres immunoreactive for vasopressin are seen in male than female rats and the normal development of this sex difference is dependent on the presence of gonadal steroids during the first two weeks of life (De Vries et al., 1985, 1986). In adult rats, the pattern of vasopressin innervation is also regulated by gonadal steroids; thus, nearly all vasopressin fibres disappear following castration, and this is reversed by treatment with T (De Vries et al., 1984). This innervation probably arises from the bed nucleus of the stria terminalis (De Vries, 1983) and a sharp decrease in the number of vasopressin containing cell bodies occurs in this area following castration of adult rats; this is reversed by treatment with T (Van Leeuwen & Caffé 1985).

Androgens also regulate release of gonadotrophins from the pituitary gland. Thus, immediately (24h) following castration of adult male rats, serum FSH is markedly increased, serum LH is also increased; no increase in pituitary FSH or LH content is detectable. A second rise in serum LH, but not FSH, occurs 7 days following castration and is accompanied by a rise in pituitary LH content (Badger et al., 1978). Both T and 5 α DHT suppress pituitary LH, but not FSH content of intact animals and oestradiol suppresses both pituitary FSH and LH content below that of vehicle treated controls (Arslan et al., 1989). Expression of the genes encoding the α subunit of gonadotrophins and the β -FSH and LH subunits is also regulated by androgens. Androgens suppress expression of the α -subunit and the β LH but not FSH subunits, whereas oestrogen administration uniformly inhibits expression of all three of the gonadotrophin subunit genes in castrates of both sexes (Gharib et al., 1987; Wierman et al., 1988).

In addition to regulating expression of the gonadotrophins, androgens and androgen metabolites also regulate the gonadotrophin releasing

hormone LHRH. Following castration of sexually mature rhesus macaques the LHRH content of the infundibular nucleus, median eminence, ventral medial nucleus, and lateral hypothalamus, are significantly reduced (Roselli et al., 1987) and these effects can be reversed by administration of either T or oestradiol (Roselli & Resko, 1990). Similar results have been found in rats (Kalra et al., 1984), in which, following castration, the proLHRH (precursor peptide of LHRH) content of the basal hypothalamus and preoptic area is reduced in a T reversible manner (Roselli et al., 1990). Expression of the LHRH gene is also regulated by T; thus, in the hypothalamus, LHRH mRNA content is markedly reduced following castration in a T reversible manner (Park et al., 1988). Given the role of LHRH in the regulation of FSH and LH it may be that some of the actions of T on these two peptides are mediated by LHRH.

In the central and medial amygdaloid nucleus of the rat a reduction both in the number of SP immunoreactive fibres, and their SP content, has been shown following castration, suggesting a trophic role of T on SP neurons in this nucleus (Dees & Kozlowski, 1984). Gonadal steroids, including T and oestradiol, are also implicated in the regulation of the development of SP immunoreactivity in the anterior pituitary of neonatal rat (Yoshikawa & Hong, 1983). However, short-term (8 day) castration of adult male rats does not significantly change SP levels in this area (Coslovsky et al., 1985; DePalatais et al., 1985), although β and γ preprotachykinin mRNA is increased (Brown et al., 1990).

SS is also under the control of T; in the median eminence, castration results in a T reversible reduction in the amount of SS, both the peptide content and the extent of peptide distribution is reduced (Gross, 1980). Interestingly, in the nearby organum vasculosum of the lamina terminalis, SS peptide is unchanged following castration. SS mRNA levels have also been found to be regulated by gonadal steroids in some (Baldino et al.,

1988; Chowen & Breed, 1989), but not all (Werner et al., 1988) areas of the CNS.

In the spinal nucleus of the bulbocavernosus (SNB), motoneurons innervating perineal striated muscles concerned with copulatory behaviour accumulate radioactive T or 5 α DHT, but not oestradiol (Breedlove & Arnold, 1980). In adult males the nucleus is found in the lumbar spinal cord, but in females is diminished or absent; its presence dependent not on genetic sex, but the action of androgens (Breedlove & Arnold, 1980). Castration of young male rats reduces the number of CGRP-immunoreactive neurons in the SNB in a T reversible manner (Popper & Micevych, 1989). Given the role of T in regulating neuropeptides including SS, SP and CGRP in a variety of tissues it may be that T regulates the neuropeptide content of *drg*. This possibility has been investigated in the rat by examining the SP, SS and CGRP content of *drg* ten days following castration or castration plus T replacement.

4.2 METHODS

Adult male Ham Wistar rats (200g) were anaesthetised under halothane and castrated or sham operated as described previously (Chapter 2). Castrated animals were split into two groups of eight and implanted subcutaneously with 30mm silastic capsules either empty or containing crystalline T (Sigma, UK). Sham operated animals received empty silastic capsules. Animals were allowed to recover and 10 days after surgery, killed by rapid decapitation, trunk blood samples collected on ice and plasma testosterone determined by RIA. *Drg* from cervical levels C4, C5, C6 and C7 were removed and the SP, SS and CGRP contents determined by RIA (Chapter 2). Results were analysed by ANOVA.

4.3 RESULTS

Following castration, plasma T concentration (Table 4.1) was below the limit of detection for the assay under the conditions used ($< 0.1 \text{ ng/ml}$). SP, SS and CGRP content of cervical *drg* following castration was unchanged compared with sham operated controls (Fig. 4.1). The model of T administration used in this study resulted in plasma T of replaced animals 13-fold greater than in sham operated controls. In animals treated with supraphysiological T, the SP, SS or CGRP content of *drg* was not significantly different from that of controls or from that of castrates.

4.4 DISCUSSION

No change in SP, SS or CGRP content of *drg* was detected following either castration or administration of supraphysiological levels of T. Previously, as discussed in the introduction to this chapter SP, SS and CGRP gene expression and peptide biosynthesis have been found to be regulated by T or its metabolites. It is possible that my chosen period of castration (10 days) did not allow sufficient time for a putative, required series of biochemical events to occur (e.g., degradation or synthesis of peptide stores) before changes in peptide content are detected. This however, is unlikely, as changes in SP in the central and medial amygdaloid nucleus are seen as early as four days following castration (Dees & Kozlowski, 1984).

That neuropeptides are regulated by T in some but not all areas of the CNS, suggests that the biochemical apparatus pre-requisite for T action may not be present in all areas of the CNS. I have discussed in the Introduction to this chapter that T can act on tissues either directly or following metabolism to 5α DHT by binding to an androgen receptor. Alternatively T may first be converted to oestrogen by aromatase and act on tissues via the oestrogen receptor. Indirect actions of T can also be mediated by T

Table 4.1

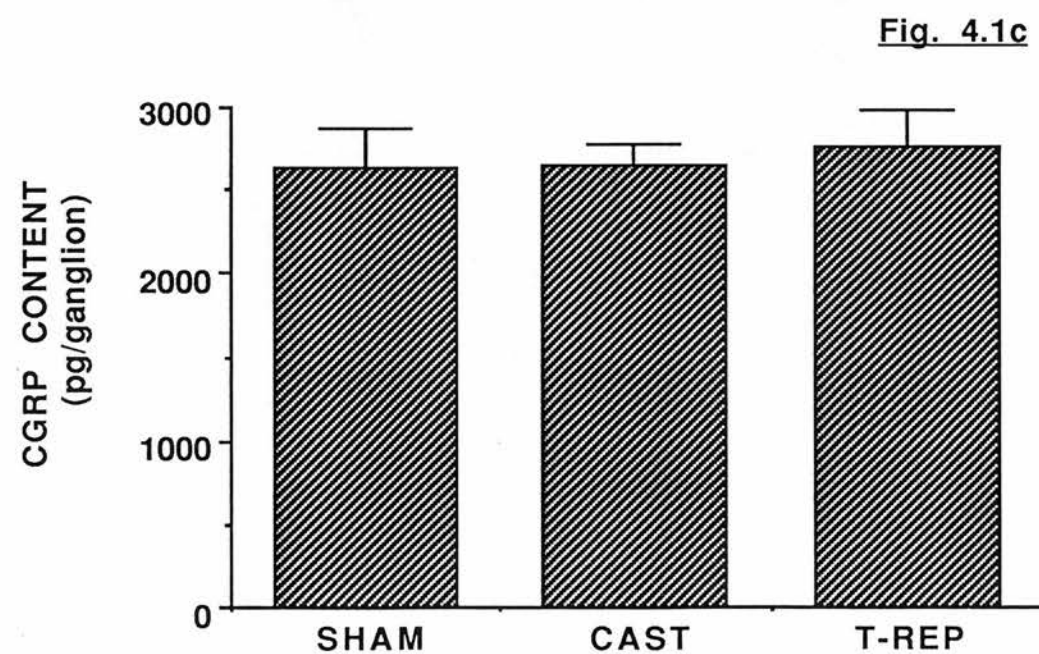
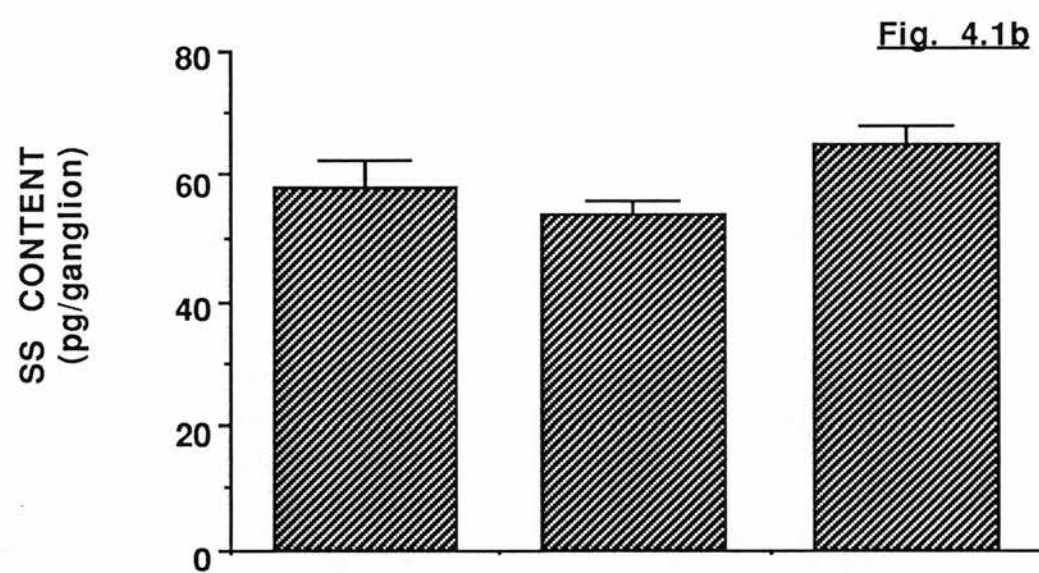
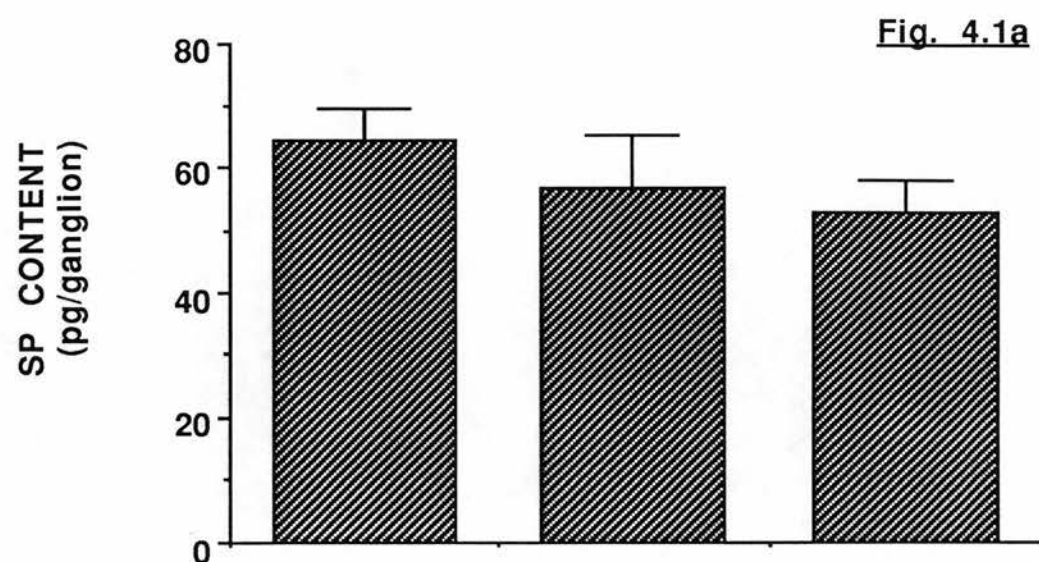
Plasma T (ng/ml) of animals following castration, sham operation or castration and administration of exogenous T

TABLE 4.1

TREATMENT	SHAM OPERATION	CASTRATION	TESTOSTERONE REPLACEMENT
Plasma Testosterone (ng/ml)	2.2 ± 0.8	< 0.1	29.2 ± 7.1

Figure 4.1

SP (a), SS (b), and CGRP (c) contents (pg/ganglion) of cervical *drg* following castration (CAST), sham operation or castration and administration of supraphysiological T (T-REP). Values are mean of pooled *drg* from level C4 to C7 with S.E.M. to bar



regulation of an intermediate factor e.g., T modulation of LHRH regulation of FSH and LH (see Introduction). Thus, for a direct action of T or its metabolites, the presence of androgen receptor and/or oestrogen receptor and associated T metabolising enzymes is an absolute requirement, although their absence does not obviate a transynaptic or some other secondary phenomenon. It is not known if these receptors and enzymes are present in *drg* of the adult male rat.

In conclusion, T does not regulate SP, SS or CGRP content of cervical *drg* in adult male rat.

CHAPTER 5

ROLE OF NEUROPEPTIDES SP, SS AND CGRP IN RAT ADJUVANT ARTHRITIS: THE EFFECTS OF ADRENALECTOMY AND GLUCOCORTICOIDS

5.1 INTRODUCTION

Numerous animal models of inflammatory disease have been developed employing a variety of mediators including collagen, carageenin and adjuvant (see Sokoloff, 1984; Magilavy, 1990). Adjuvant arthritis, a chronic, non-infectious polyarthritis was developed largely by Pearson and colleagues (1956) and occurs in certain strains of rats, following intraplantar injection of Freund's complete adjuvant (FCA). Adjuvant disease is evoked by injection of bacterial peptidoglycans contained in water-in-oil emulsions, usually derived from mycobacteria, but peptidoglycan from nocardia, corynebacteria and other gram positive organisms are also effective (Hadler & Granovetter, 1978). A number of mechanisms have been suggested to account for the adjuvant function of these bacterial fractions. Current opinion holds that the adjuvant stimulates an immune response in T and B cells and macrophages, however N-acetylmuramyl-L-alanyl-D-isoglutamine is a non-antigenic synthetic analogue which is both arthritogenic and an adjuvant (Kohashi et al., 1982). Thus, it may be that the presence of D isomers, as well as the usual L-isomer of amino acids in peptidoglycans, may represent a catabolic problem for macrophages, although not all peptidoglycans have adjuvant activity. It has been suggested that the use of an oil in water emulsion reflects the importance of the physico-chemical properties of the peptidoglycans in conferring adjuvant activity, but streptococcal peptidoglycans produce adjuvant like lesions without prior emulsification. Finally, CP-20961 a synthetic fat-soluble, non-immunogenic non-peptidoglycan compound also induces an arthritis in rats comparable to that of adjuvant disease (Chang et al., 1980). Despite the fact that the mechanisms mediating adjuvant arthritis remain elusive it has become widely employed as a model of inflammatory disease.

Adjuvant disease (Rosenthale & Capetola, 1982) is a painful systemic disorder affecting almost exclusively the distal portions of the extremities:

the carpals and tarsals and portions of the paws beyond them. Following injection of isotopically labelled mycobacteria into the tail base (polyarthritis), radioactivity is found in many parts of the body, with only a small proportion in the hind paws (Glenn et al., 1977). Initially, an acute, peri-arthritis, characterised by inflammation of periarticular tissues and tendon sheaths occurs following a lag period. The synovial spaces are infiltrated with predominately lymphocytes, monocytes and macrophages, although some polymorphonuclear leukocytes are also found. Subsequently, hyperplasia of synovial lining cells, and proliferation of granulation tissue occur, and later subchondral bone and cartilage destruction ensue accompanied by florid production of new bone in the adjacent periosteum. Residual ankylosis and exophytic bone remain after the inflammatory process recedes (see Sokoloff, 1984). It is also possible to produce a more localised inflammation (monoarthritis) by injection of FCA into particular tissues. In such animals, a similar inflammatory process occurs to that found in joints of polyarthritic animals, but the inflammatory processes are localised to the injected area.

Thus, conditions similar to those encountered clinically in RA are manifest following injection of FCA into the tail base or peri-articular tissue of rats. A number of studies have examined the role of neuropeptides in the aetiology of adjuvant disease employing both polyarthritic and monoarthritic animals. Weihe and colleagues (1988) used immunohistochemistry to investigate changes in neuropeptides of lumbosacral spinal cord and *drg* in a polyarthritis and hind paw monoarthritis. In lumbosacral dorsal horn of polyarthritic animals a moderate increase in staining for SP and CGRP immunoreactive fibres in laminae I, II and V was noted, compared with untreated controls. A similar moderate intensification of CGRP and SP immunoreactivity in the dorsal horn on the ipsilateral compared to the contralateral side of monoarthritic animals was also observed, and

immunostaining for CGRP in cells of the *drg* was more intense; no increase in the number of SP and CGRP producing cells was noted. Interestingly, in the inflamed joint, SP and CGRP immunoreactivity was elevated compared with untreated controls. The increase in CGRP immunoreactivity was particularly pronounced, accompanied by apparent sprouting of fibres within the epidermis, subepidermal regions and in the vicinity of subepidermal venules. CGRP immunoreactive fibres were also found associated with mast cells and immunocompetent cells and in areas of infiltrated immune cells.

Treatment with capsaicin, an irritant extracted from peppers, depletes neuropeptides including SP. Capsaicin treatment has also been shown to reduce the inflammatory response to adjuvant (Colpaert et al., 1983). In studies of capsaicin-evoked neuropeptide release from dorsal slices of lumbar spinal cord from adjuvant arthritic rats inoculated in both hind paws with FCA, release of CGRP was significantly enhanced compared with controls (Nanayama et al., 1989). CGRP content was also increased in lumbosacral *drg* (Kuraishi et al., 1989) of arthritic rats, and immunisation of rats against CGRP reduces the inflammatory response to inoculation with adjuvant (Louis et al., 1990).

A role for SP in the pathology of adjuvant arthritis has been suggested from a number of studies. In the lumbar ventral spinal cord of polyarthritic animals, Chery-Croze et al. (1985) reported the SP concentration to be reduced, but no change in dorsal cord SP content was reported. In contrast, Lembeck et al. (1981) reported a significant increase in SP content of dorsal cord from L4-L5. More recently, spontaneous release of SP into the spinal cord was found to be enhanced in polyarthritic rats over controls. Furthermore, passive movement of inflamed joints potentiated spontaneous release of SP but in control animals, no increase in SP release was apparent following passive movement, suggesting that

SP was directly involved in the transmission to the dorsal horn of pain, produced by the movement (Oku et al., 1987a). Supporting the role of SP in inflammation, it has been shown that the density of innervation by SP-containing primary afferents is correlated with the severity of arthritis that joint develops. Thus, in the adjuvant rat model, severely arthritic joints (tarsals) have a more dense SPergic innervation than more mildly arthritic joints (knees) (Levine et al., 1984). In addition, infusion of SP into the inflamed joint potentiated the arthritis and SP antagonists ameliorated the inflammation. Lembeck (1981) reported SP to be increased in *drg*, but not significantly, following induction of polyarthritis. Recently, Satoh (1990) and Ohno (1990) have reported increased SP, CGRP and SS content of *drg* in adjuvant and carageenin models of inflammatory disease.

Given the role of neuropeptides in pain and inflammation, and the previously described regulation of neuropeptides by glucocorticoids (Chapter 3), I have investigated the changes in SP, SS and CGRP content of *drg* of adjuvant-induced arthritic rats and examined the effect of ADX and glucocorticoids on the progression of inflammation and neuropeptide content of *drg*.

5.2 METHODS

Under halothane anaesthesia, Ham Wistar rats (male, 300g) were injected subdermally around the left carpal joint with either Freund's complete adjuvant (FCA; Sigma, UK) or incomplete adjuvant (ICA; Sigma, UK). Two subdermal injections each of 0.05ml were made at different locations around the carpal joint of the forepaw and the animals allowed to recover. To assess the progress of the inflammation, the body weight and the circumference of the carpal joints were recorded for the duration of the experiments. A small tape measure was placed around the joint and the circumference determined.

In a preliminary experiment (Experiment 1), Ham Wistar rats (male, 250-300g) were divided into two groups ($n = 5$) and one group received FCA as described; the second group received ICA and served as controls. Twenty-two days following injection, the animals were killed by decapitation, *drg* on ipsilateral and contralateral sides dissected from level C4 to C7 and frozen on dry ice. The pooled *drg* were subsequently homogenised in 2M acetic acid and SS, SP and CGRP content determined by RIA; results were expressed in pg/ganglion. For comparison between treatments an unpaired t-test was used, and for comparison between ipsilateral and contralateral sides, a paired t-test was used.

A second experiment (Experiment 2) was performed to chart more accurately the *drg* in which peptide contents changed. In this second experiment, three groups ($n = 5$) of Ham Wistar rats (male, 250-300g) received FCA, ICA or no treatment. Fifteen days after induction of adjuvant arthritis, the animals were killed by decapitation, and trunk blood samples collected in order that plasma B and ACTH contents could be measured by RIA. On ipsilateral and contralateral sides of the spinal cord, *drg* were dissected and collected in pairs from level C2 to T6. The tissues were prepared for RIA and SP, SS and CGRP contents determined as described previously and expressed in pg/ganglion. For comparison between treatment groups, ANOVA was used with Duncan's multiple range test. For comparison between ipsilateral and contralateral sides, repeated measures ANOVA, followed by Scheffe's F-test was used.

A third and final experiment (Experiment 3) was concerned with the effects of ADX and glucocorticoids on adjuvant-induced inflammation and neuropeptide changes. Ham Wistar rats (male, 250-300g) were divided into two groups. One group ($n = 7$) received injection of ICA around the carpal joint; the remaining animals were injected with FCA. Ten days following injection of FCA, animals in this group were subdivided into a further five

groups. Some animals were bilaterally adrenalectomised ($n = 10$), using a single step procedure, or sham operated ($n = 7$). A further two groups of animals were orally administered DEX (0.2mg/kg; $n = 7$) or RU38486 (10mg/kg; $n = 7$) in saline, respectively. The drugs were given daily in 0.9% saline, 0.2% ethanol by gavage (0.4ml/100g) until the experiment was terminated. The remaining group of animals given FCA received no further treatment. The experiment was terminated 20 days after the peri-articular injection of FCA or ICA. Animals were killed by decapitation and plasma samples collected and frozen for determination of B and ACTH content. *Drg* from the ipsilateral and contralateral sides were dissected in pairs between level C2 and T6, frozen on dry ice, and stored at -20°C . The tissues were subsequently homogenised in 2M acetic acid, SP and CGRP content determined by RIA and expressed in pg/ganglion. Statistics were used as described for experiment 2.

5.3 RESULTS

5.3.1 Experiment 1

Animals were injected unilaterally with FCA and sacrificed 22 days later. SP, SS and CGRP content of pooled *drg* from ipsilateral and contralateral sides, of level C4 to C7, were determined.

Body Weight

It is apparent that in this experiment, a slight difference in the body weight of the two groups existed from the start. Animals receiving FCA lost some weight compared with those receiving ICA immediately following injection, but this did not reach significance (Fig 5.1., ANOVA). There was no difference in the rate of weight gain between the two groups

Carpal joint Circumference

Injection of FCA or ICA produced significant swelling of the ipsilateral carpal joint compared with the contralateral carpal joint (Fig. 5.2b) between

Figure 5.1 The body weight (gm) of animals following peri-articular injection of FCA (0.1mg) or ICA. Values are mean with S.E.M. to bar

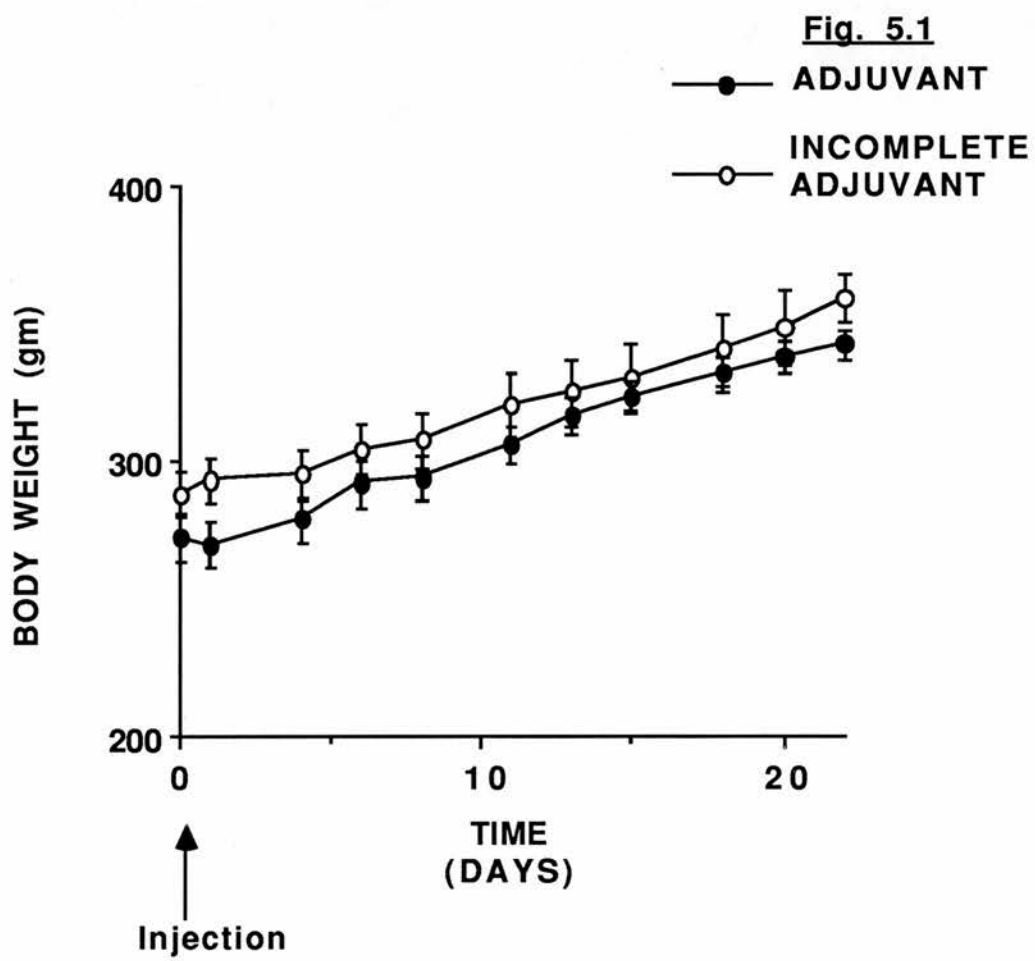


Figure 5.2

The circumference (cm) of the ipsilateral (a) and contralateral (b) carpal joints of rats after injection of FCA or ICA. Values are mean with S.E.M. to bar.

There was significantly more inflammation in the forepaw of animals following injection of FCA compared with ICA

(* $p < 0.05$, ** $p < 0.01$, c.f. ipsilateral joint of ICA treated animals; ANOVA, Duncan's Multiple Range Test)

Fig. 5.2a

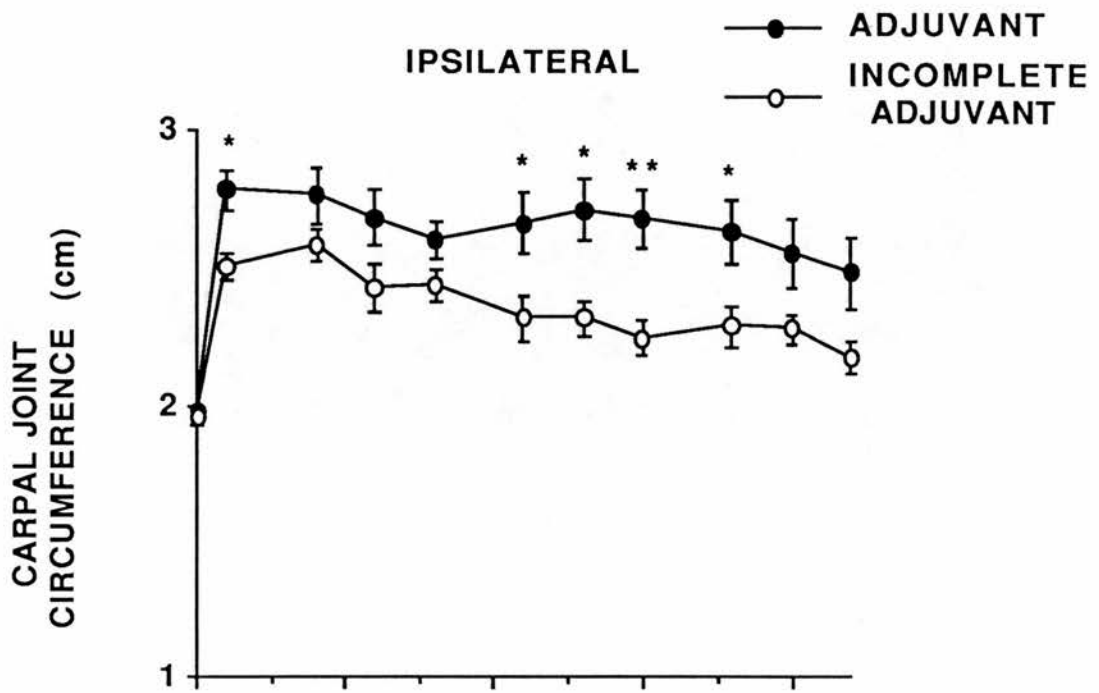
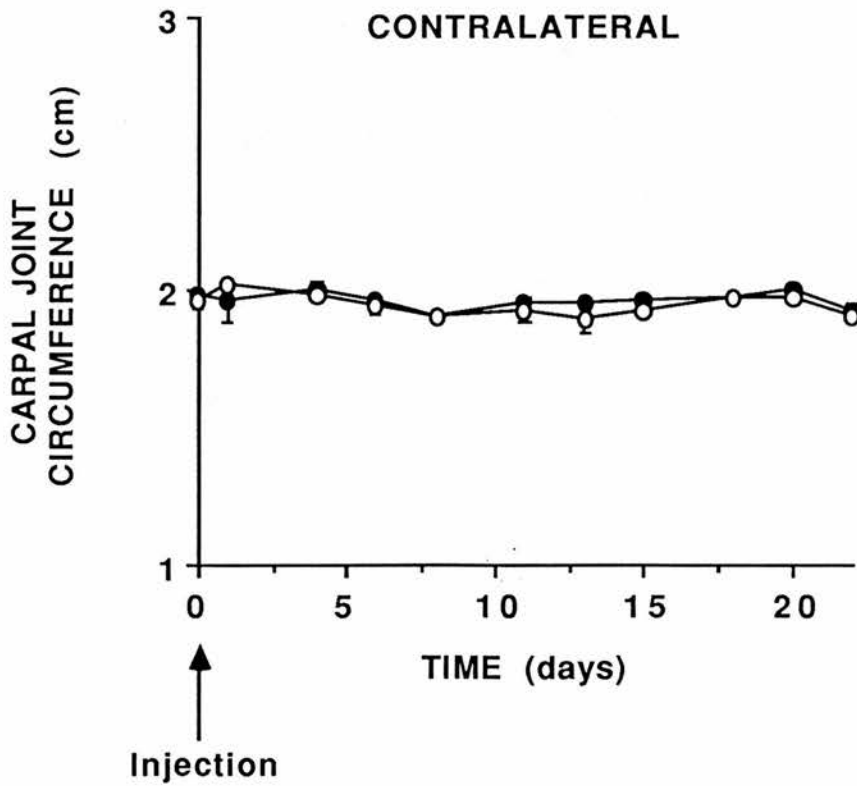


Fig. 5.2b



day 1 and 22 ($p < 0.01$; ANOVA, Scheffe's F-test). This swelling was more pronounced in the FCA-treated animals than those in the ICA group.

Neuropeptide Contents

There was no significant difference between the SS, SP or CGRP content of ipsilateral and contralateral *drg* of animals injected with ICA (Fig. 5.3). Following injection of FCA, no significant difference in the SS content was detected; whilst the CGRP content did tend to be greater, this did not reach significance. In contrast, the SP content of ipsilateral *drg* was significantly greater than that of contralateral *drg*. There was a tendency for SP content of contralateral *drg* to be lower in FCA-treated animals compared with ICA-injected animals; this difference did not, however, reach significance (unpaired t-test).

5.3.2 Experiment 2

In order to more accurately define the *drg* in which neuropeptide changes occurred, adjuvant arthritis was induced and animals sacrificed 15 days later. SP, SS and CGRP content of pairs of *drg*, from ipsilateral and contralateral sides, at level C2/3, C4/5, C6/7, T1/2, T3/4 and T5/6 were determined.

Body weight

There was no significant difference in the body weight of the three groups at any time during the experiment (Fig. 5.4) .

Carpal joint circumference

In ICA and FCA injected animals, the ipsilateral carpal joint was significantly swollen one day after injection compared with the untreated animals; this swelling persisted for the duration of the experiment (Fig. 5.5a). FCA injection produced a significantly more pronounced and prolonged inflammation than ICA. No significant difference in the

Figure 5.3

SS (a), SP (b) and CGRP (c) contents (pg/ganglion) of pooled *drg* collected from ipsilateral and contralateral level C4 to C7 of animals 22 days following unilateral injection of FCA or ICA. Values given are mean with S.E.M. to bar
(*p <0.05; paired t-test)

Fig. 5.3a

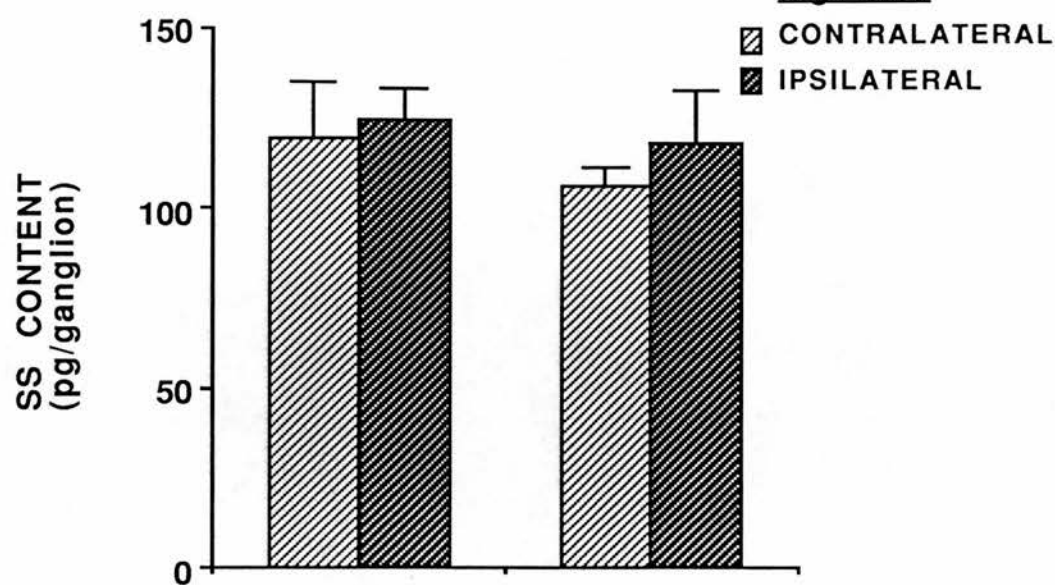


Fig. 5.3b

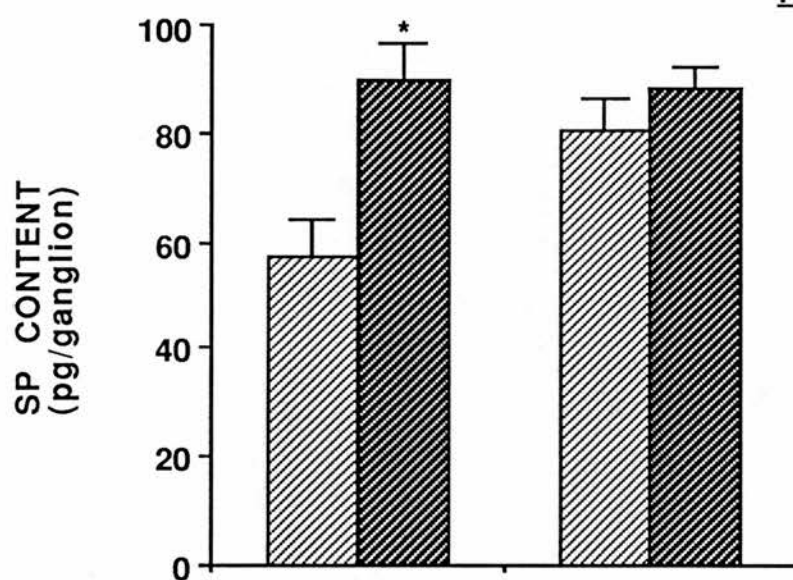


Fig. 5.3c

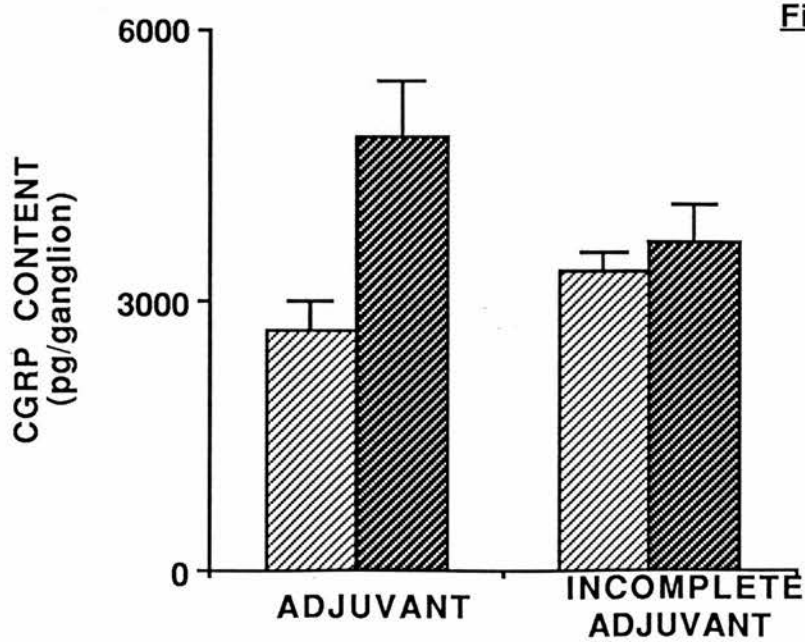


Figure 5.4 The body weight (gm) of animals following no treatment (N/T) or subdermal injection of FCA or ICA to the left carpal joint. Values are mean with S.E.M. to bar

Fig. 5.4

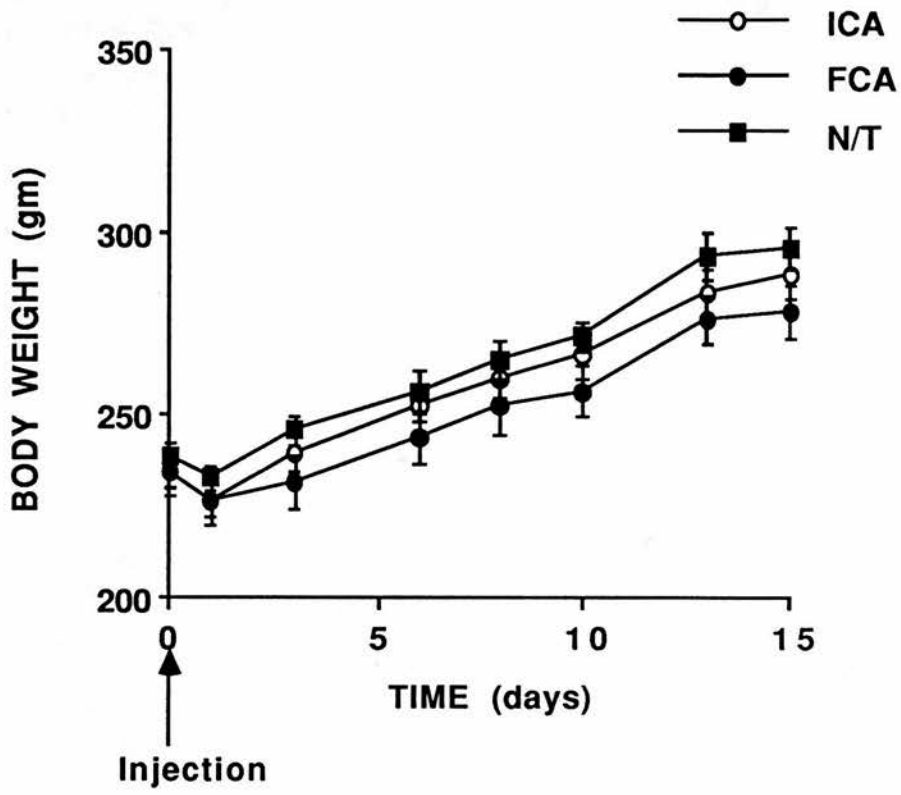


Figure 5.5

The circumference (cm) of the ipsilateral (a) and contralateral (b) carpal joint of animals after receiving no treatment (N/T) or peri-articular injection of FCA or ICA. Values are mean with S.E.M. to bar (**p < 0.01, c.f. ipsilateral joint of untreated animals, ANOVA, Duncan's Multiple Range Test)

Fig. 5.5a

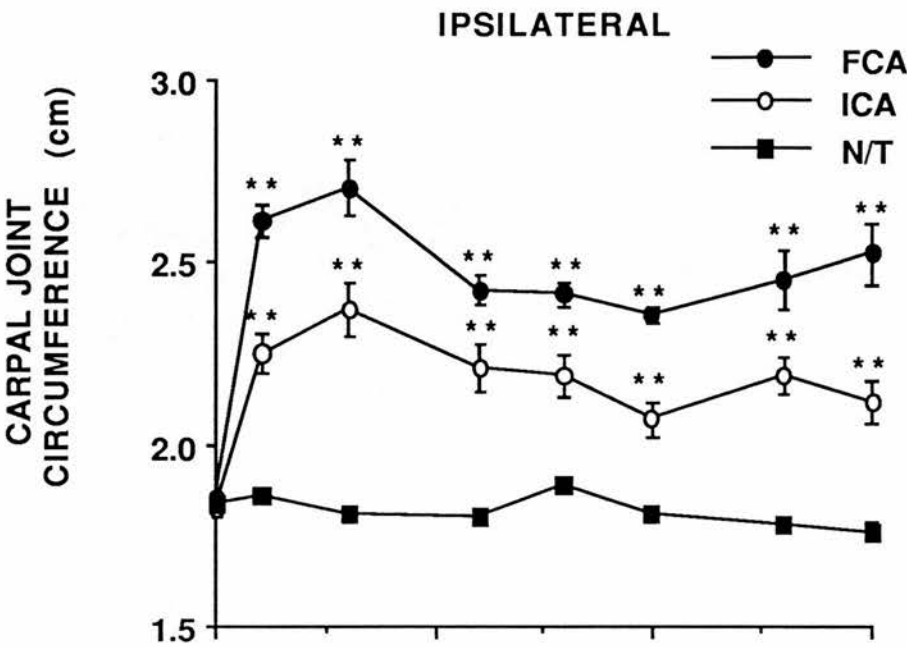
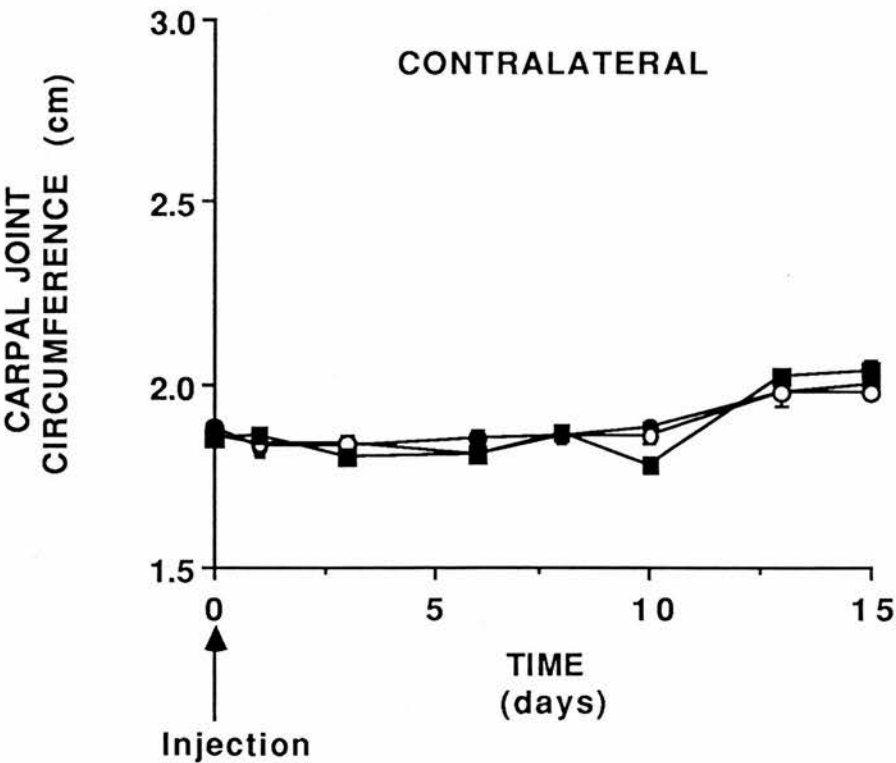


Fig. 5.5b



circumference of the contralateral carpal joint between any of the groups was detected throughout the experiment (Fig. 5.5b).

Plasma Data

There was no significant difference in plasma B between ICA-treated animals and those receiving no treatment (Table 5.1). However, plasma B was significantly greater 15 days following injection of FCA compared with ICA injected animals or animals receiving no treatment. No significant difference in plasma ACTH was detected between the three groups.

Neuropeptide Contents

In untreated animals, SS content (Fig. 5.6) of *drg* from contralateral and ipsilateral sides was greatest at C2/3 and least in *drg* from C6/7 and T5/6. This distribution did not change 15 days following peri-articular injection of ICA or FCA. There was no significant difference in SS content of ipsilateral *drg* compared with the contralateral side in any of the three treatment groups (ANOVA).

In untreated animals, SP content (Fig. 5.7) of contralateral and ipsilateral *drg* was greatest in *drg* from level C6/7, and decreased rostrally and caudally. Fifteen days following subdermal injection of ICA or FCA, the SP content of both contralateral and ipsilateral *drg* was similarly distributed to that of untreated animals. There were no significant differences between the SP content of *drg* from ICA, FCA or untreated animals at any of the levels studied, either on the ipsilateral or contralateral side (ANOVA), and within each group the SP content of ipsilateral *drg* did not differ significantly from that of contralateral *drg*, although SP content of ipsilateral C6/7 *drg* of FCA treated animals did tend to be greater than the contralateral.

On the contralateral and ipsilateral sides of untreated animals CGRP content (Fig. 5.8) was greater in cervical than thoracic *drg*. The distribution of CGRP in *drg* from both ipsilateral and contralateral sides in either FCA- or ICA-treated animals was similar to that in untreated animals. CGRP content

Table 5.1 Plasma B (ng/ml) and ACTH (pg/ml) of untreated animals and animals 15 days following injection of FCA or ICA. Values given are mean \pm S.E.M. (*p < 0.05, **p < 0.05, c.f., FCA-treated animals, unpaired t-test)

TABLE 5.1

	FCA	ICA	N/T
Plasma Corticosterone (ng/ml)	286.9 ± 53.2	105.5 ± 22.0**	133.6 ± 31.1*
Plasma ACTH (pg/ml)	119.0 ± 56.0	63.6 ± 6.0	166.9 ± 94

Figure 5.6 SS content (pg/ganglion) of *drg* collected in pairs C2/3, C4/5, C6/7, T1/2, T3/4 and T5/6 from ipsilateral and contralateral sides of untreated animals (a) and animals 15 days following subdermal injection of ICA (b), or FCA (c). Values are mean with S.E.M. to bar

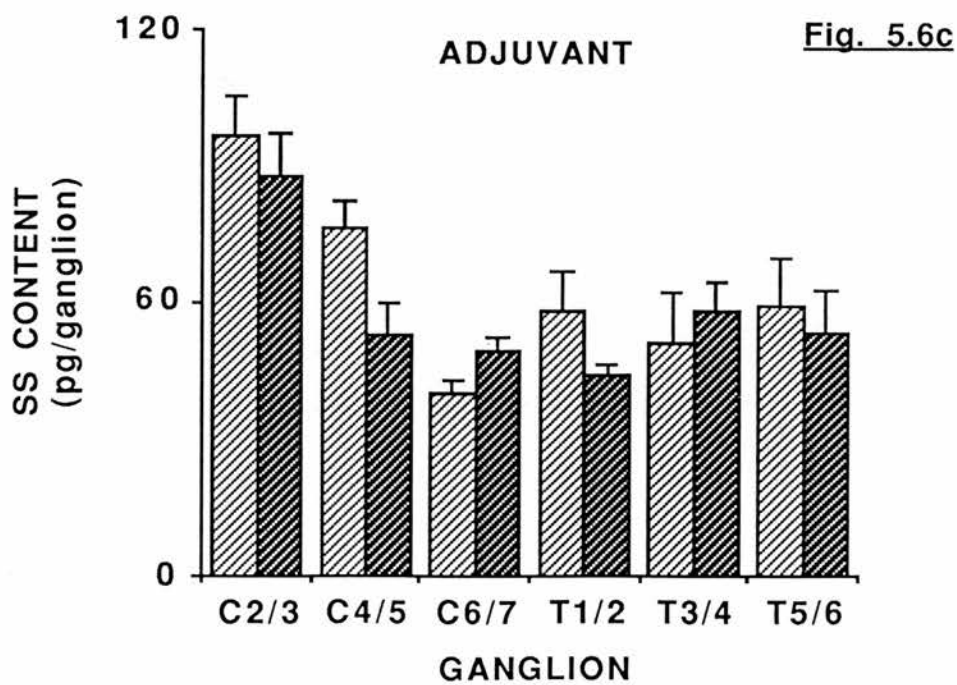
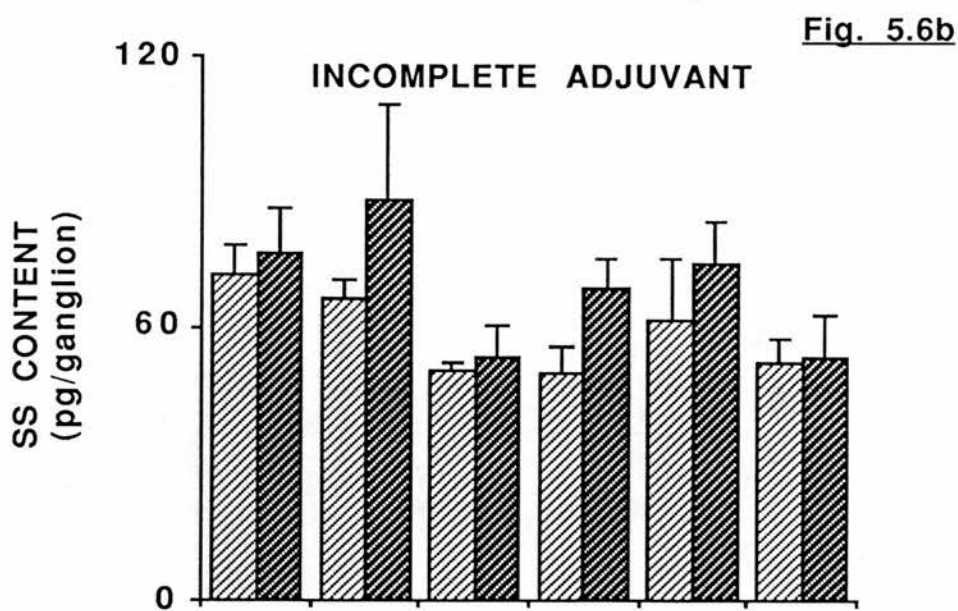
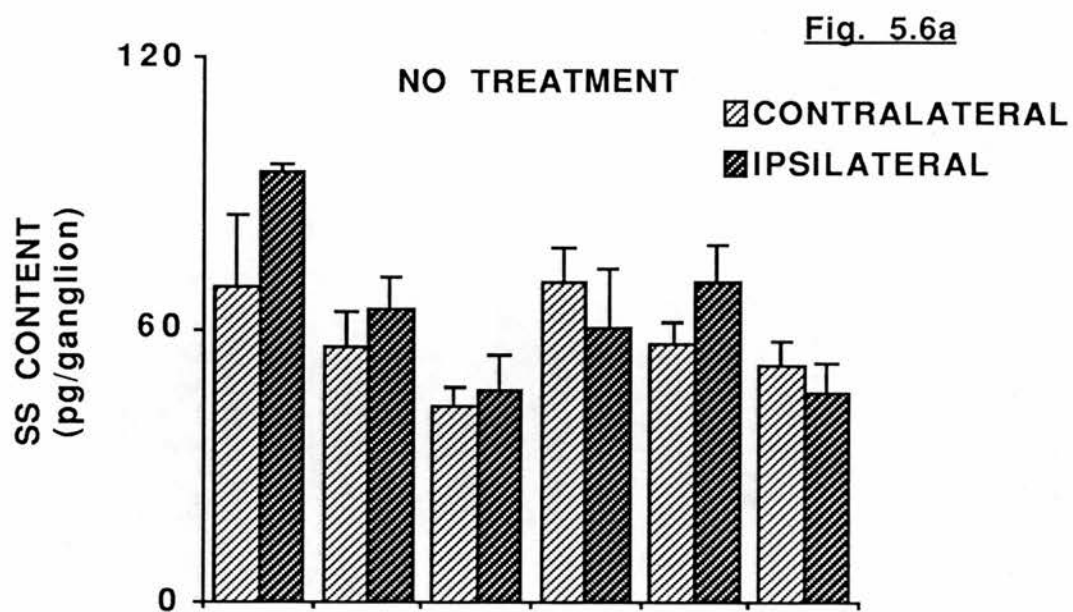


Figure 5.7 SP content (pg/ganglion) of *drg* collected in pairs C2/3, C4/5, C6/7, T1/2, T3/4 and T5/6 of ipsilateral and contralateral sides of untreated animals (a) and animals 15 days following subdermal injection of ICA (b) or FCA (c). Values are mean with S.E.M. to bar

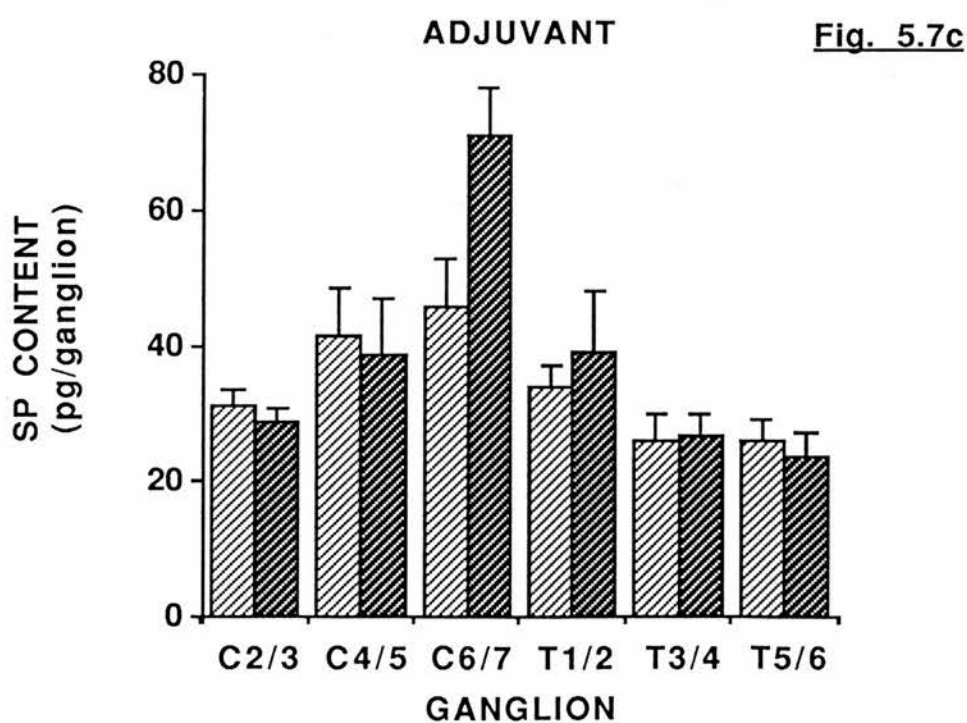
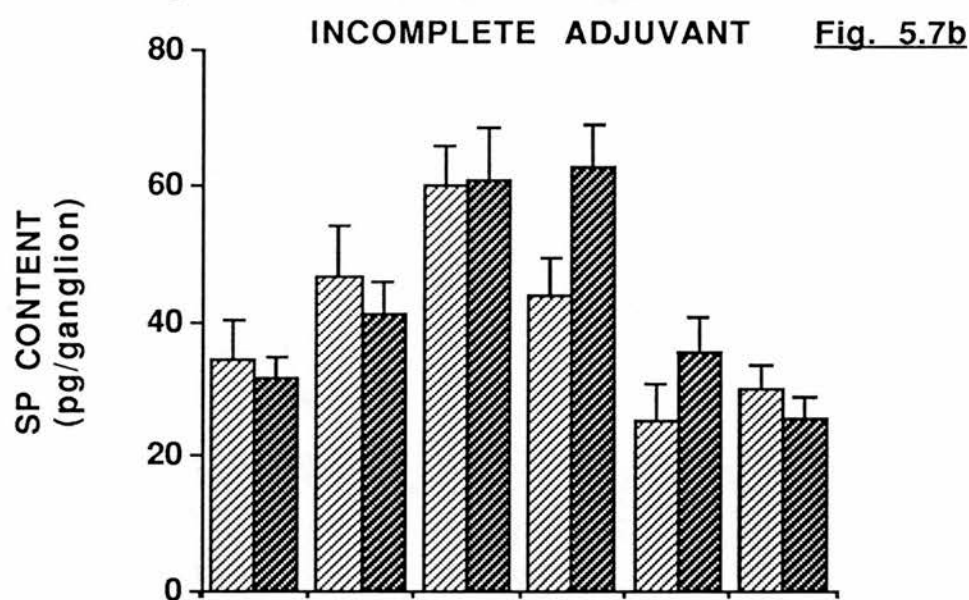
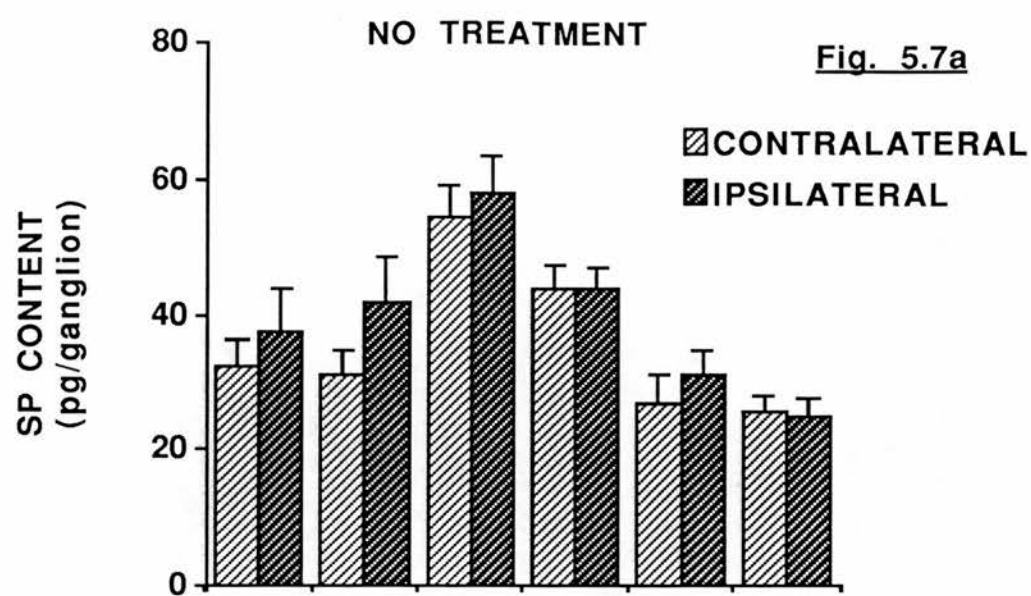


Figure 5.8 CGRP content (pg/ganglion) of *drg* collected in pairs C2/3, C4/5, C6/7, T1/2, T3/4 and T5/6 of ipsilateral and contralateral sides of untreated animals (a) and animals 15 days following subdermal injection of ICA (b) or FCA (c). Values are mean with S.E.M. to bar (*p < 0.05, **p < 0.01, c.f. contralateral side, ANOVA, Scheffe's F-test)

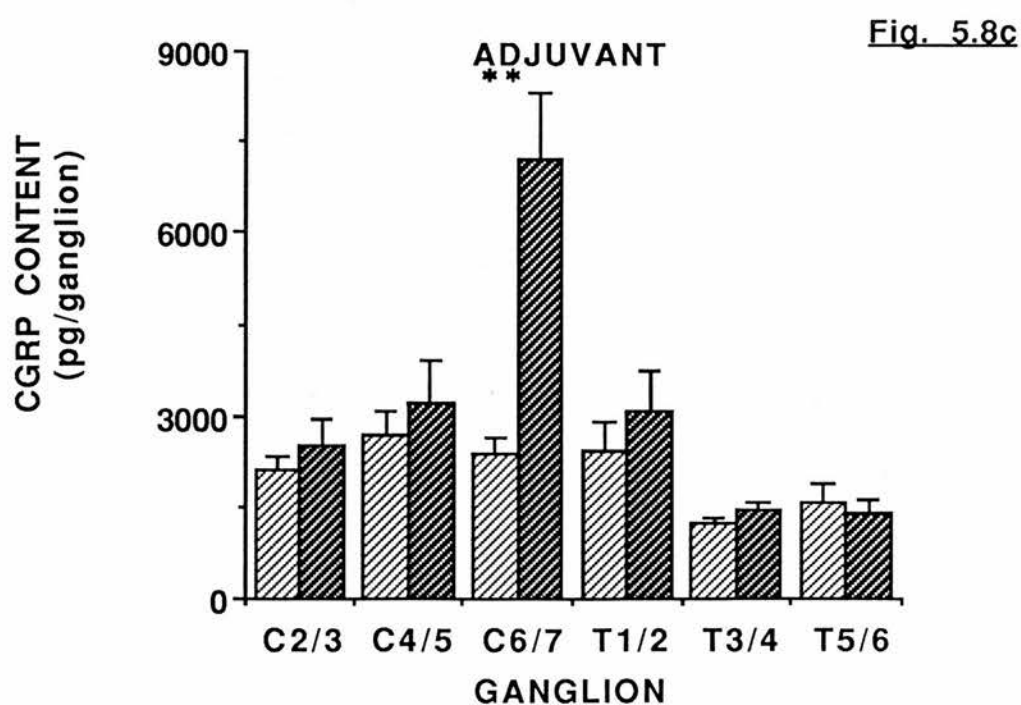
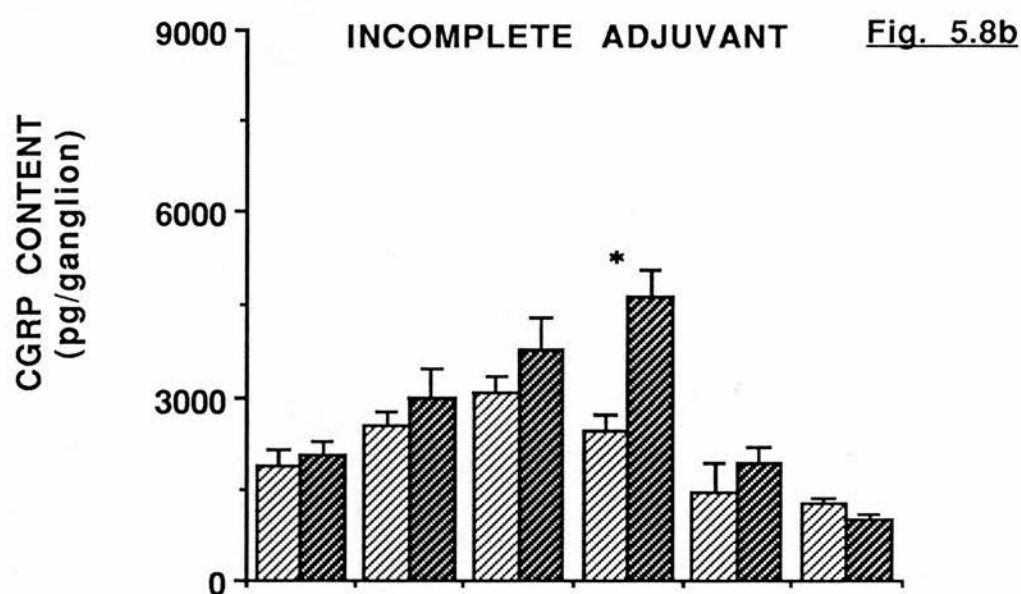
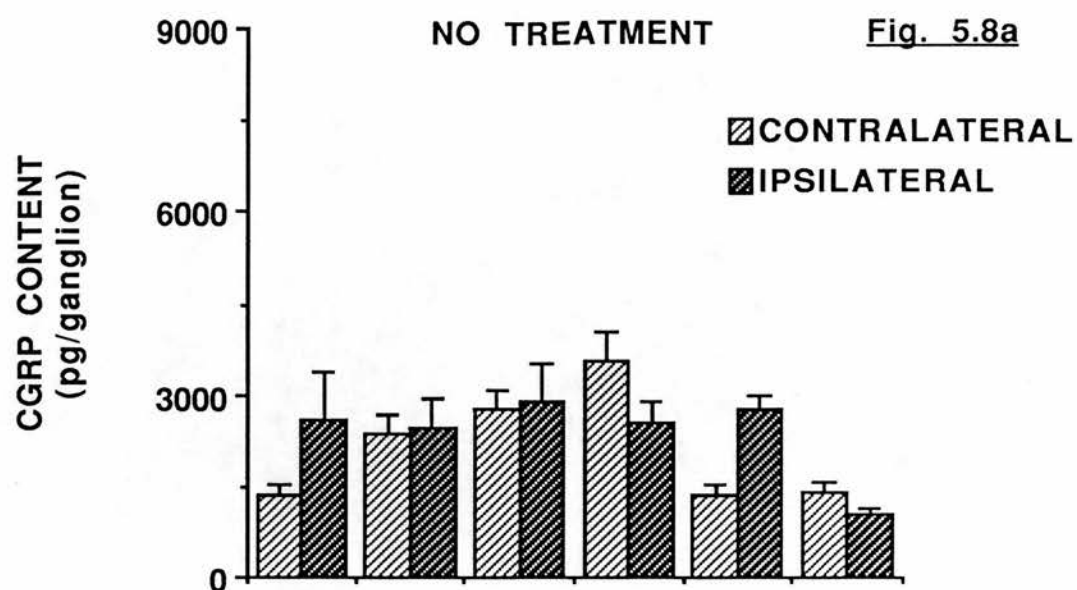


Figure 5.9a Body weight (gm) of animals following subdermal injection of FCA or ICA. Values are mean with S.E.M. to bar

Figure 5.9b Body weight (gm) of animals following subdermal injection of FCA and either adrenalectomy or sham operation. Values are mean with S.E.M. to bar

Figure 5.9c Body weight (gm) of animals following subdermal injection of FCA, and subdermal injection of FCA with daily dose of RU486 or DEX. Values are mean with S.E.M. to bar
(**p < 0.01, c.f. FCA group, ANOVA, Duncan's Multiple Range Test)

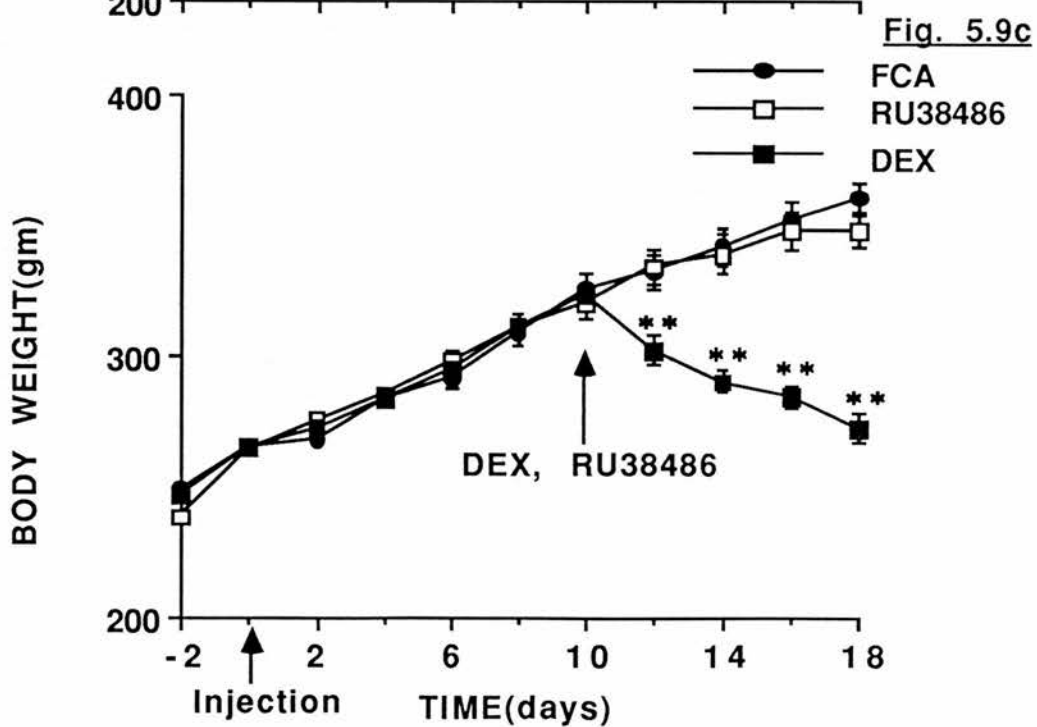
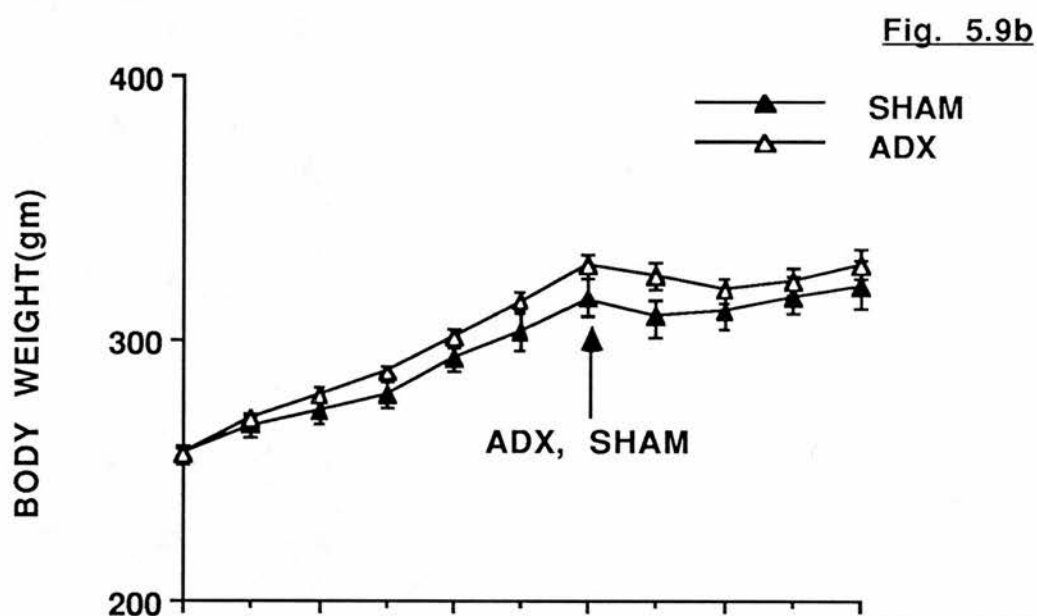
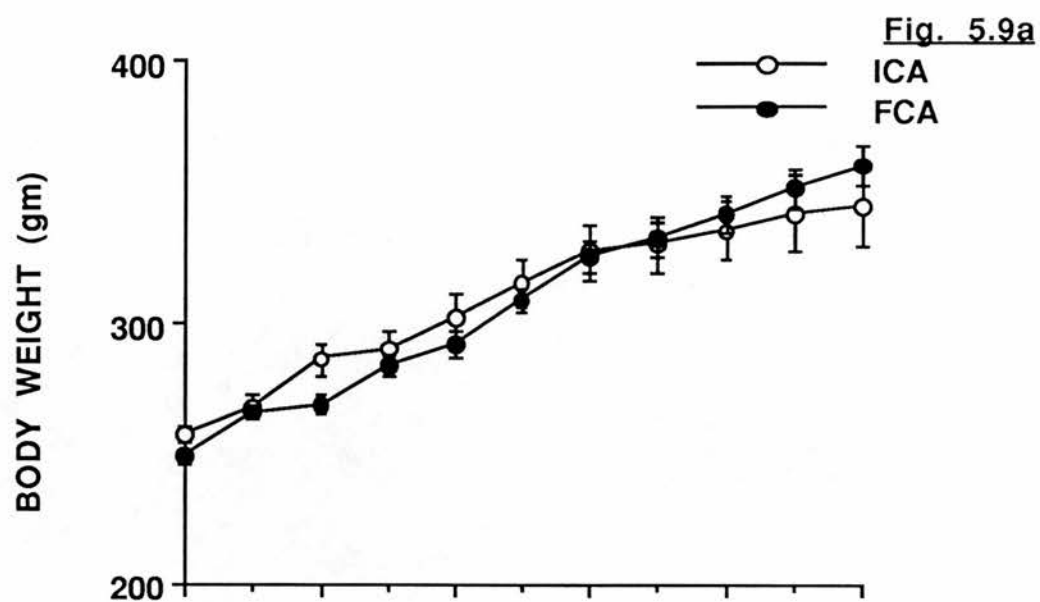


Figure 5.10 The circumference (cm) of the ipsilateral (a) and contralateral (b) carpal joint of rats following injection of FCA or ICA. Values are mean with S.E.M. to bar (* $p < 0.05$, ** $p < 0.01$, c.f. ICA injection, ANOVA, Duncan's Multiple Range Test)

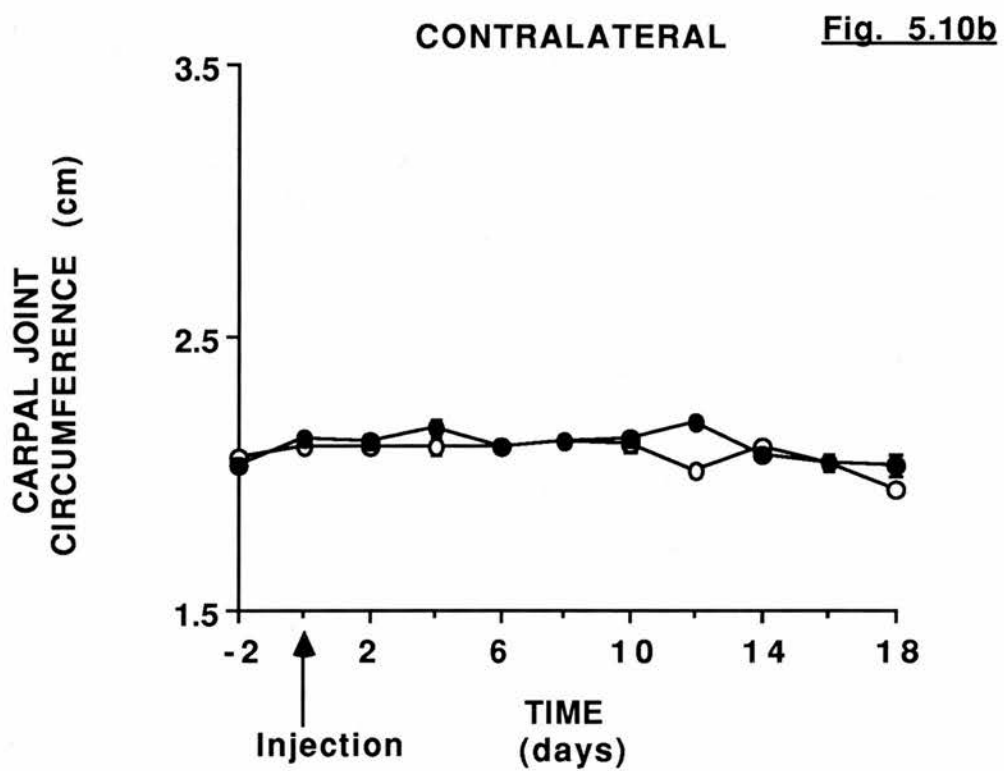
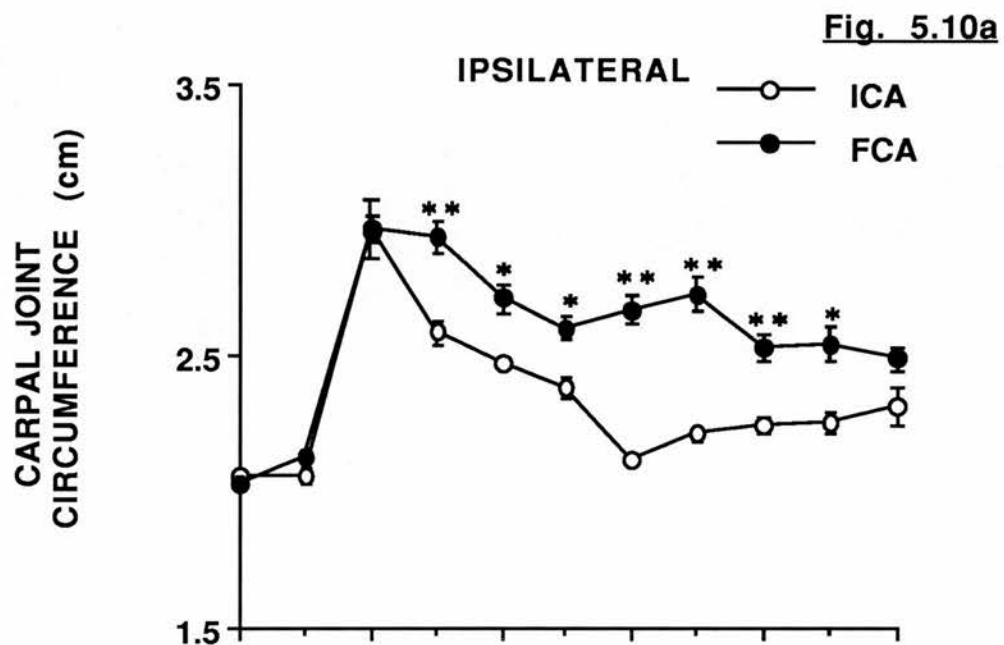


Table 5.2

Plasma B (ng/ml) and ACTH (pg/ml) of animals in each of the six treatment groups; plasma samples were collected at the termination of the experiment. Values given are mean \pm S.E.M.

TABLE 5.2

	FCA	ICA	SHAM	ADX	DEX	RU38486
Plasma Corticosterone (ng/ml)	176.6 ± 19.4	89.2 ± 43.5	112.7 ± 19.6	< 2.9	< 2.9	219.6 ± 41.2
Plasma ACTH (pg/ml)	140.2 ± 59.0	75.2 ± 25.6	53.4 ± 11.0	581.8 ± 216.6	68.6 ± 17.0	81.3 ± 13.8

of ipsilateral C6/7 *drg* in FCA-treated animals was significantly ($p < 0.01$; ANOVA, Duncan's Multiple Range Test) greater than that in untreated or ICA-injected animals and was significantly greater than that of the contralateral side of the same animals. Similarly, the CGRP content of ipsilateral T1/2 *drg* of ICA-injected animals was significantly greater ($p < 0.05$) than the CGRP content of *drg* from the same area in untreated or FCA-treated animals and was also significantly greater than that in the contralateral side of the same animals.

5.3.3. Experiment 3

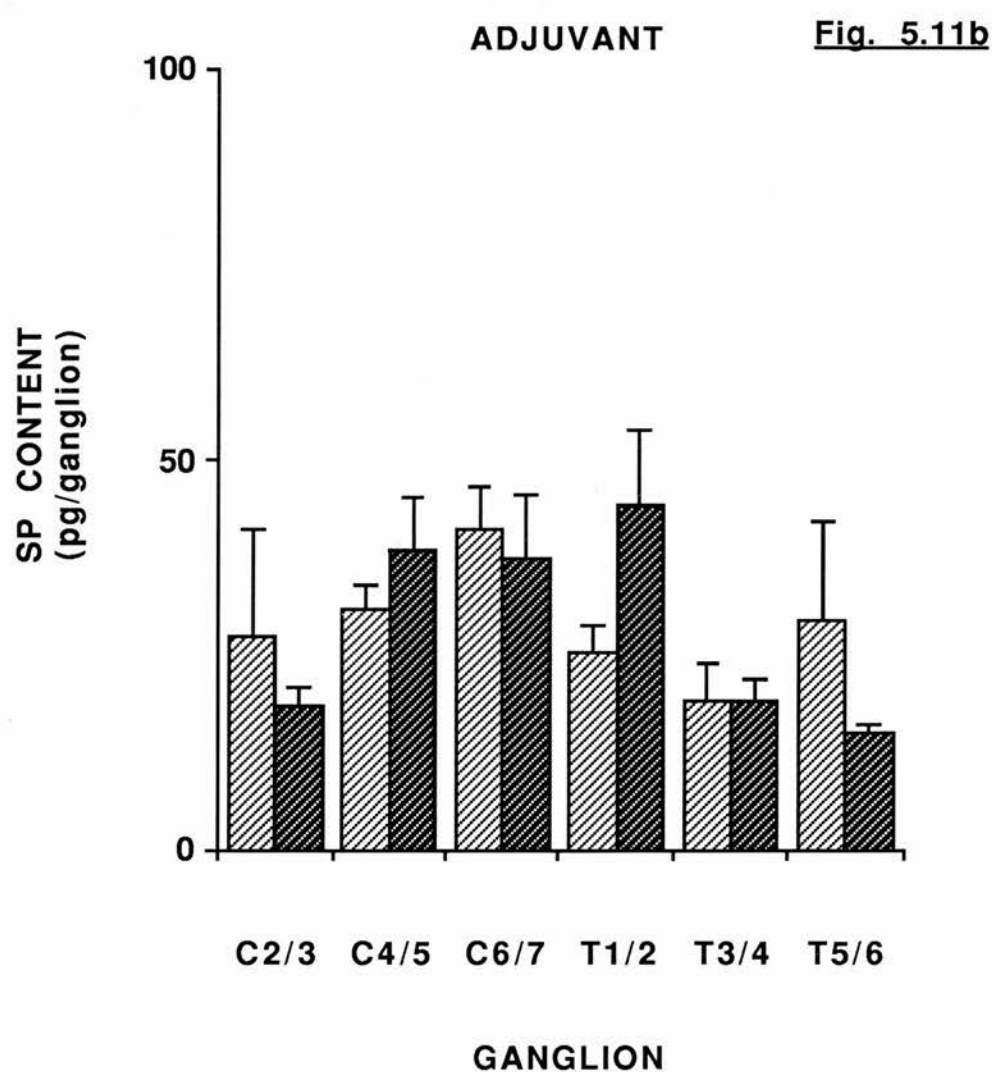
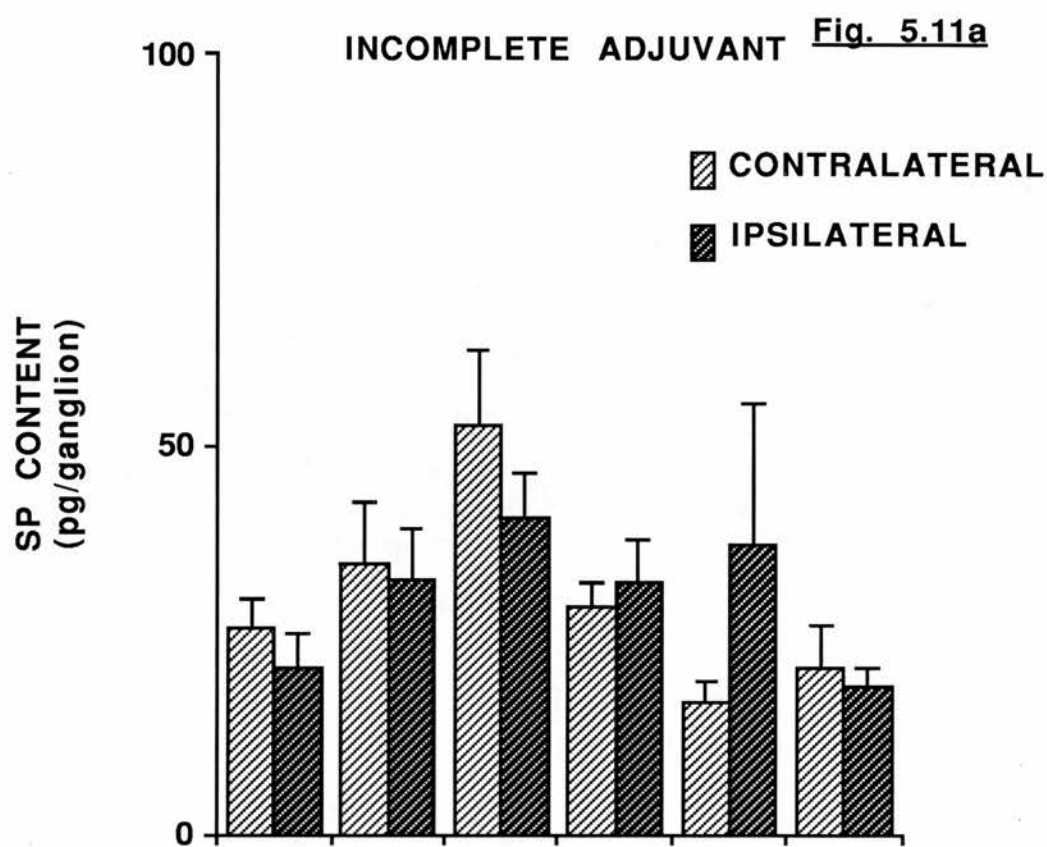
Animals were administered adjuvant and 10 days later, animals were adrenalectomised or sham operated or daily treatment with DEX or RU38486 commenced. A further 10 days later, animals were sacrificed and SP and CGRP content of pairs of *drg* from C2/3 to T5/6 of ipsilateral and contralateral sides determined.

Effects of FCA and ICA

There was no significant difference (ANOVA) in the body weights of FCA- or ICA-treated animals at any time during the experiment (Fig. 5.9a). As in previous experiments, injection of either ICA or FCA produced a pronounced swelling of the ipsilateral carpal joint (Fig. 5.10a), which was significantly greater in the FCA treated group. Whilst plasma B and ACTH of FCA-treated animals (Table 5.2) tended to be greater than that of ICA-treated animals, this difference did not reach significance (unpaired t-test).

The SP content of *drg* from C2/3 to T5/6 of FCA- and ICA-treated animals is given in Fig. 5.11. The distribution of SP was broadly similar to that found previously in Experiment 2. Comparing the SP content of *drg* from C2/3 to T5/6 between the two treatment groups, no significant difference in SP content in any of the areas studied was noted (ANOVA).

Figure 5.11 SP content (pg/ganglion) of *drg* collected in pairs C2/3, C4/5, C6.7, T1/2, T3/4 and T5/6 from ipsilateral and contralateral sides of animals injection with ICA (a) or, FCA (b). Values are mean with S.E.M. to bar



Similarly, in both the ICA- and FCA-treated groups, no significant difference between ipsilateral and contralateral SP content was found.

The distribution of CGRP content (Fig. 5.12) of *drg* from level C2/3 to T5/6 of FCA or ICA treated animals was similar to that found previously in Experiment 2. Comparing the CGRP content of *drg* from C2/3 to T5/6 between the two groups, no significant difference in the content of the two groups was noted (ANOVA). Similarly, but in contrast to Experiment 2, no significant difference in CGRP content of ipsilateral compared with contralateral *drg* was found in either ICA or FCA treated animals.

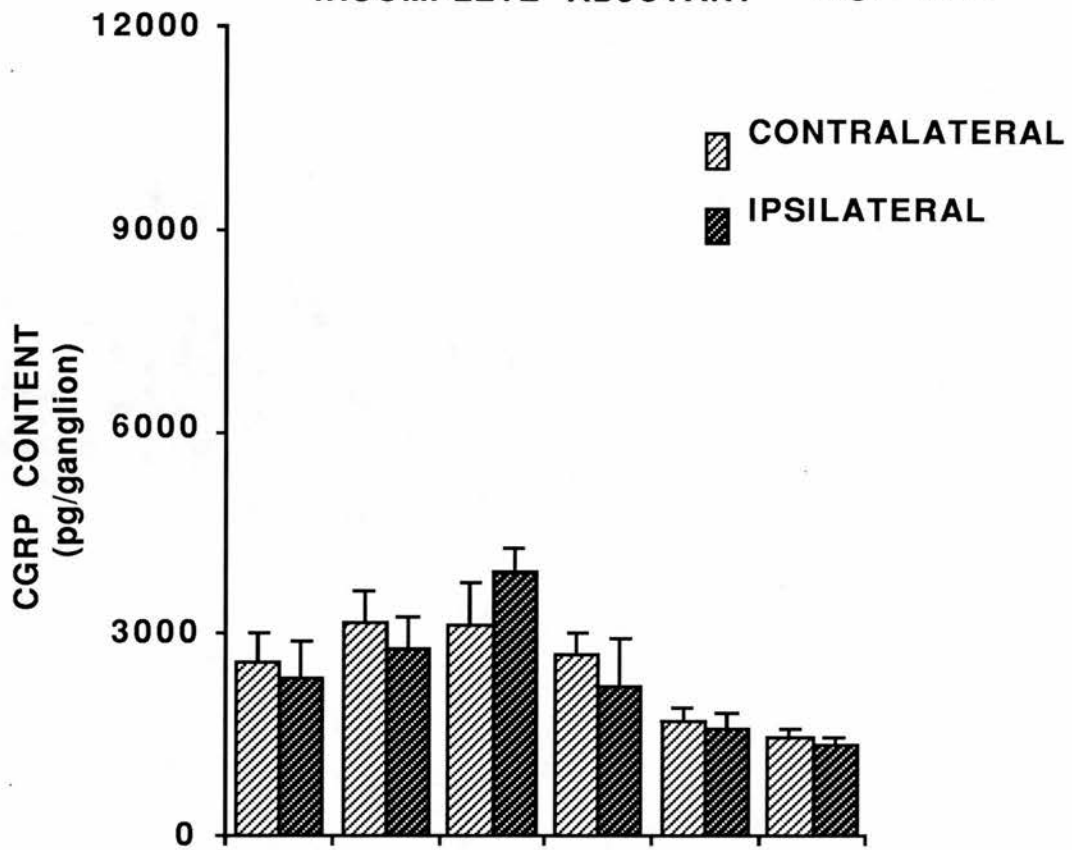
Effects of ADX or Sham operation on FCA-injected animals

The body weight of adrenalectomised animals was not significantly different from that of sham operated controls throughout the duration of the experiment (ANOVA, Fig. 5.9b); whilst both treatments did tend to inhibit the weight gain found in FCA- or ICA-treated animals, this was not significant. As expected, following ADX, plasma B (Table 5.2) was below the limit of sensitivity of the assay, but sham operation did not alter plasma B compared with unoperated animals (FCA-treated group). Similarly, as expected following ADX, plasma ACTH increased above that of sham operated animals (Table 5.2). For the initial 10 days of the experiment prior to ADX, no significant difference in the circumference of the ipsilateral carpal joint between the two groups was noted (Fig. 5.13). Following ADX (on day 10), the circumference of the ipsilateral forepaw of animals in this group was significantly greater than that of sham operated controls.

No significant difference in the SP content of *drg* from level C2/3-T5/6 was apparent between sham operated (Fig. 5.14a) or adrenalectomised animals (Fig. 5.14b; ANOVA). Similarly, the SP content of ipsilateral *drg* in either the sham operated or adrenalectomised animals did not differ significantly from that of the contralateral at any level studied (ANOVA).

Figure 5.12 CGRP content (pg/ganglion) of *drg* collected in pairs C2/3, C4/5, C6/7, T1/2, T3/4 and T5/6 from ipsilateral and contralateral sides of animals injected with ICA (a) or, FCA (b). Values are mean with S.E.M. to bar

INCOMPLETE ADJUVANT Fig. 5.12a



ADJUVANT Fig. 5.12b

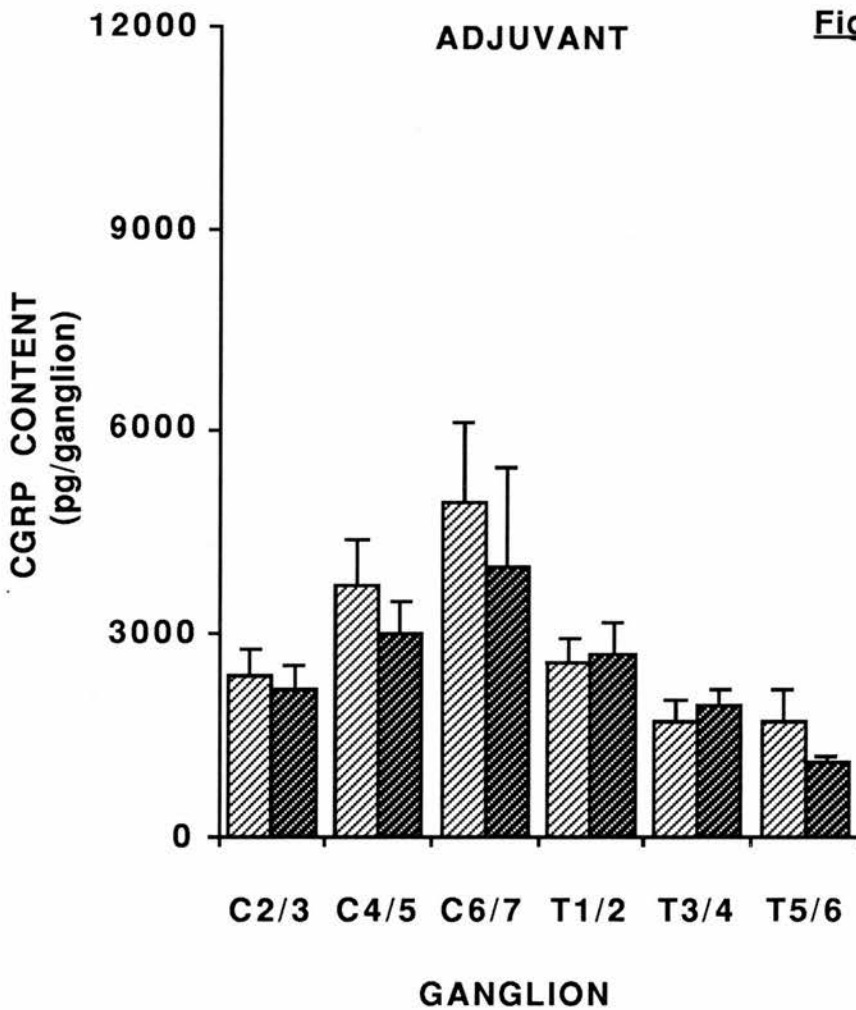


Figure 5.13 The circumference (cm) of the ipsilateral (a) and contralateral (b) carpal joint of rats following subdermal injection of FCA and either sham operation or ADX. Values are mean with S.E.M. to bar (*p < 0.05 c.f. sham operation; ANOVA, Duncan's Multiple Range Test)

Fig. 5.13a

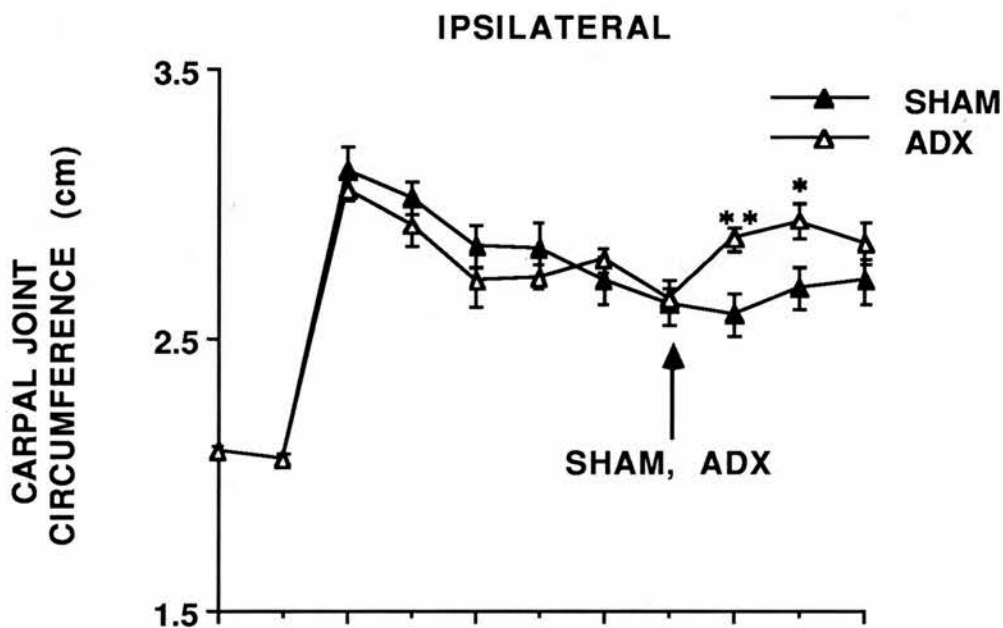


Fig. 5.13b

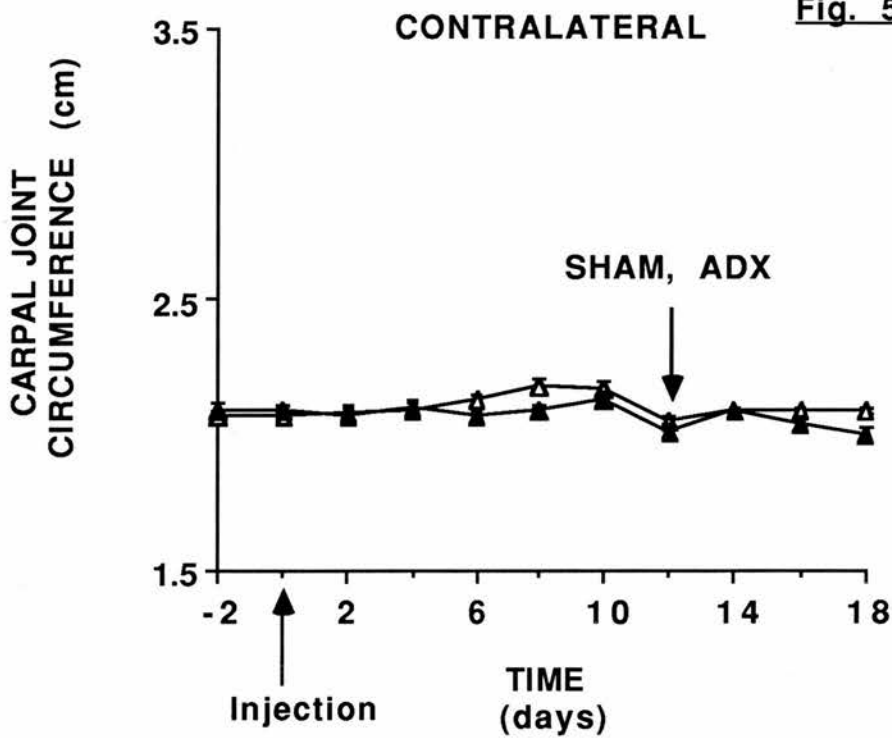
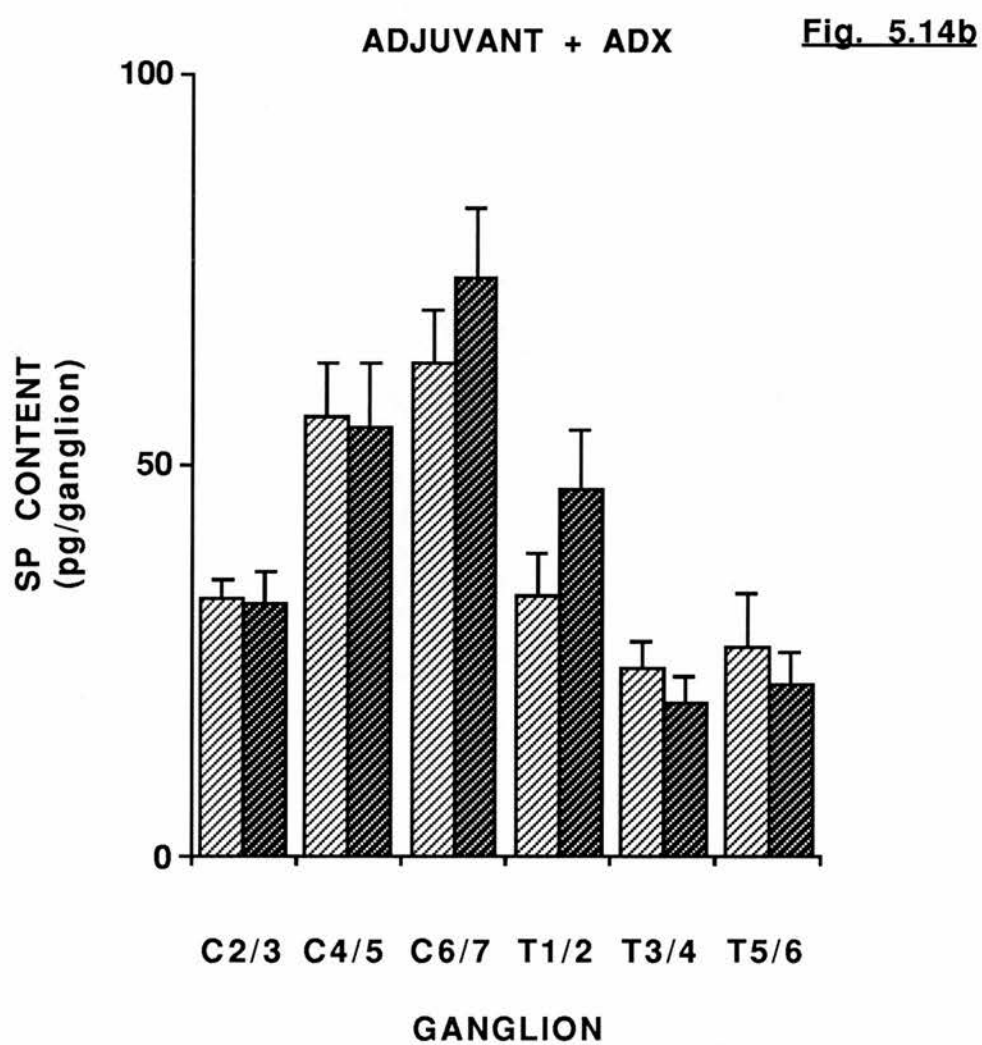
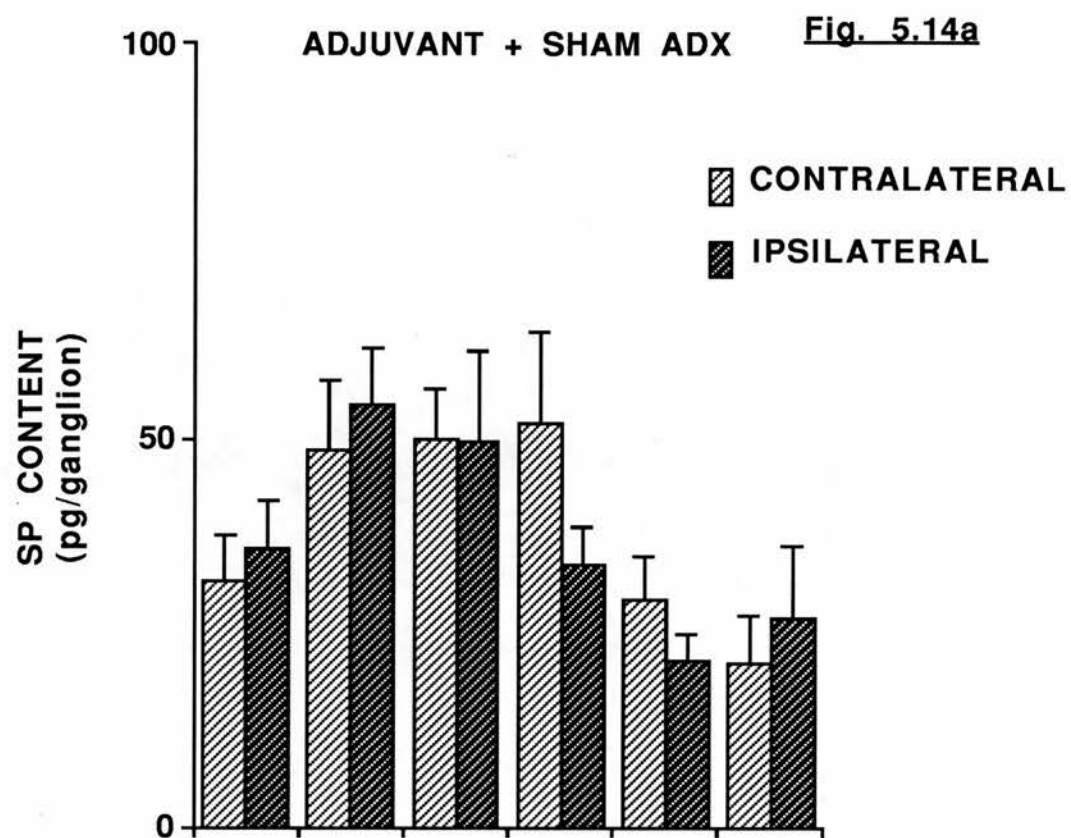


Figure 5.14 SP content (pg/ganglion) of *drg* collected in pairs C2/3, C4/5, C6/7, T1/2, T3/4 and T5/6 from ipsilateral and contralateral sides animals injected with FCA and sham operated (a) or, adrenalectomised (b). Values are mean with S.E.M. to bar



Following ADX, CGRP content (Fig. 5.15) of ipsilateral C6/7 *drg* was significantly greater than that of sham operated animals ($p < 0.01$; ANOVA, Duncan's multiple range test), and of contralateral C6/7 *drg*. Similarly, in area T1/2, the CGRP content of ipsilateral *drg* of adrenalectomised animals was significantly greater than the contralateral side and that of sham operated controls ($p < 0.01$).

Effects of DEX on FCA-injected animals

Treatment of animals with DEX from day 10 to day 20 produced a significant fall in body weight (Fig. 5.9c) compared with FCA-treated controls. Treatment of animals with DEX suppressed plasma B below the limit of detection of the assay and significantly below that of FCA-treated controls and reduced plasma ACTH significantly below that of FCA-treated controls (Table 5.2). Up until day 10 (the commencement of DEX treatment), no significant difference between the circumference of the ipsilateral carpal joint of the two FCA-treated groups was detected (Fig. 5.16). Following DEX treatment, the circumference of the ipsilateral carpal joint fell compared to that of the FCA-treated controls (day 12 - day 18). Thus, DEX treatment ameliorated the inflammation caused by FCA, such that by day 18 the ipsilateral forepaw circumference was not significantly different from that at day 0.

Whilst DEX treatment tended to reduce SP content of *drg* from all areas compared to animals receiving FCA alone, this was not significant (ANOVA, Fig. 5.17). The SP content of ipsilateral C6/7 *drg* from DEX-treated animals, was significantly greater than that of the contralateral. No other differences between ipsilateral and contralateral SP contents were detected.

The CGRP content of *drg* from DEX-treated animals, tended in all areas to be less than that of animals receiving FCA alone, but again, this was not significant (ANOVA; Fig. 5.18). Similarly, comparison of the CGRP

Figure 5.15 CGRP content (pg/ganglion) of *drg* collected in pairs C2/3, C4/5, C6/7, T1/2, T3/4 and T5/6 from ipsilateral and contralateral sides of animals injected with FCA and sham operated (a) or, adrenalectomised (b). Values are mean with S.E.M. to bar (*p < 0.05, **p < 0.01; ANOVA, Scheffe's F-test)

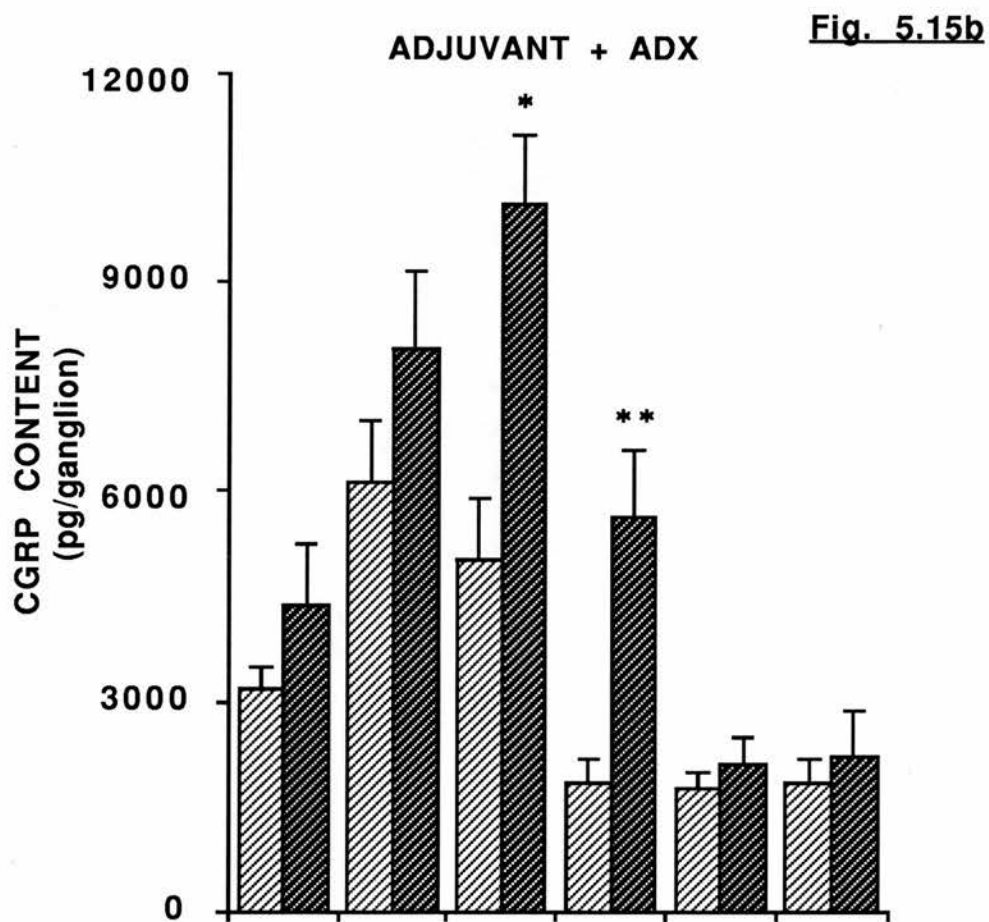
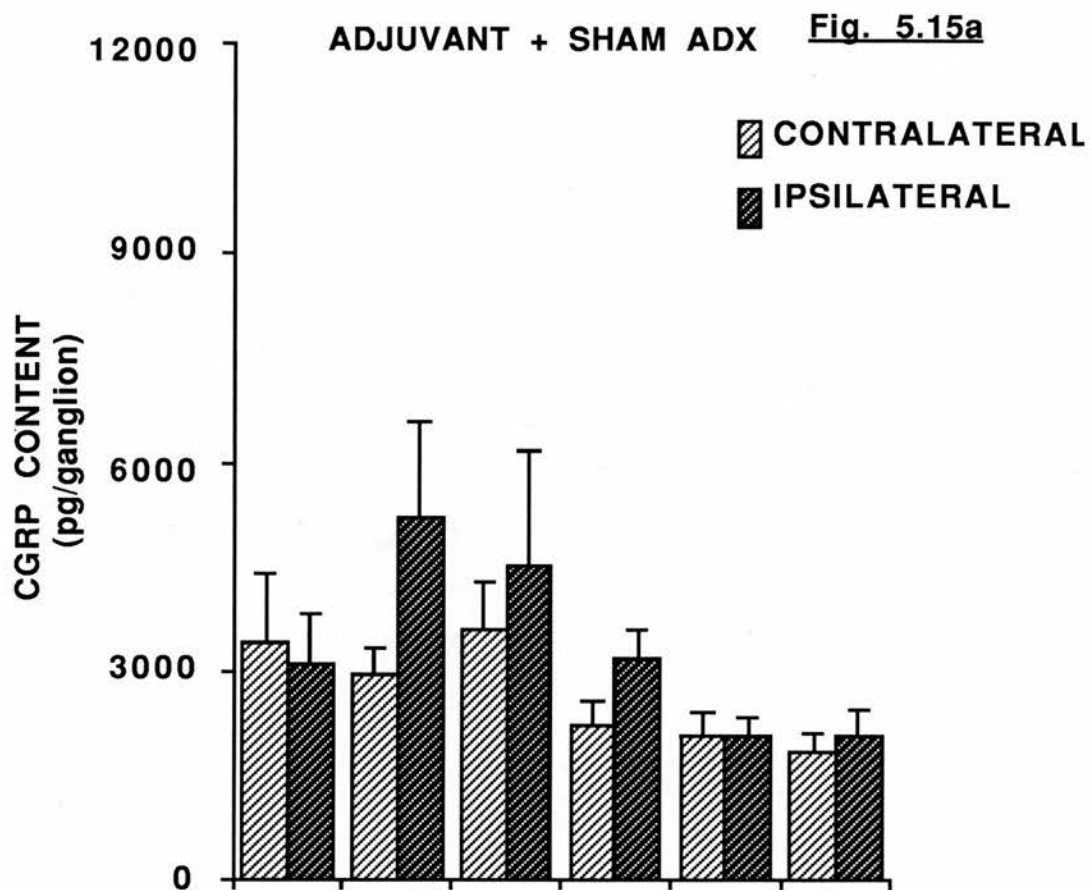


Figure 5.16 The circumference (cm) of the ipsilateral (a) and contralateral (b) carpal joint of rats receiving FCA injection and vehicle, DEX or RU38486. Values are mean with S.E.M. to bar
(**p < 0.01 c.f. vehicle treatment; ANOVA, Duncan's Multiple Range Test)

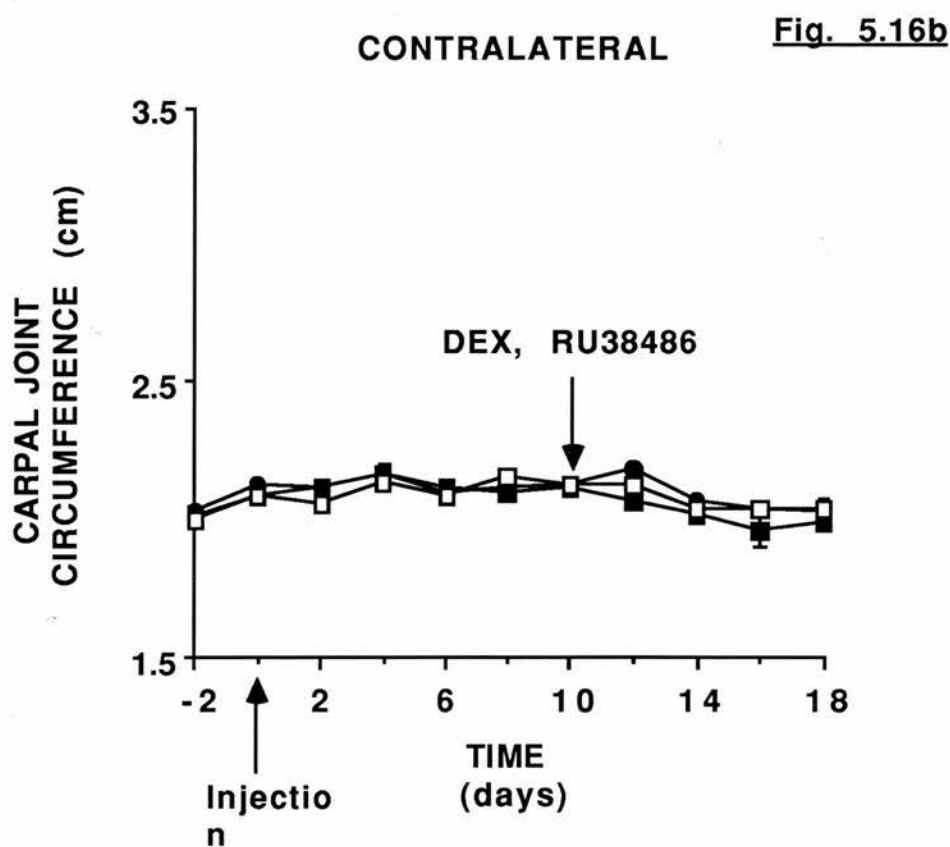
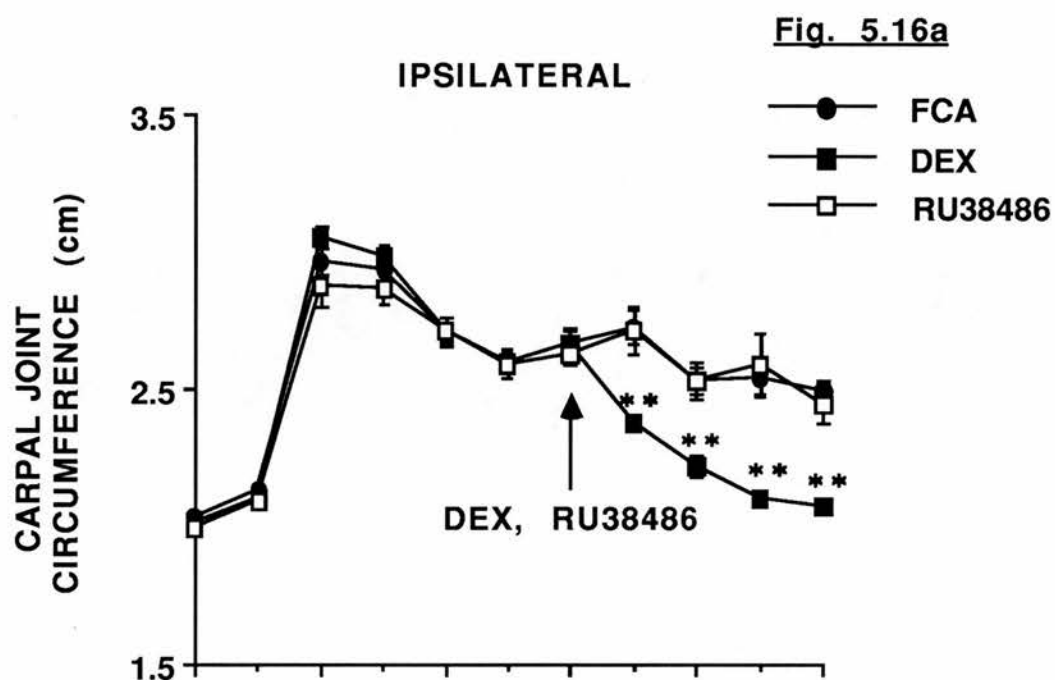


Figure 5.17 SP content (pg/ganglion) of *drg* collected in pairs C2/3, C4/5, C6/7, T1/2, T3/4, and T5/6 from ipsilateral and contralateral sides of animals injected with FCA and administered DEX (a) or RU38486 (b) from days 10 to 20. Values are mean with S.E.M. to bar (*p < 0.05; ANOVA, Scheffe's F-test)

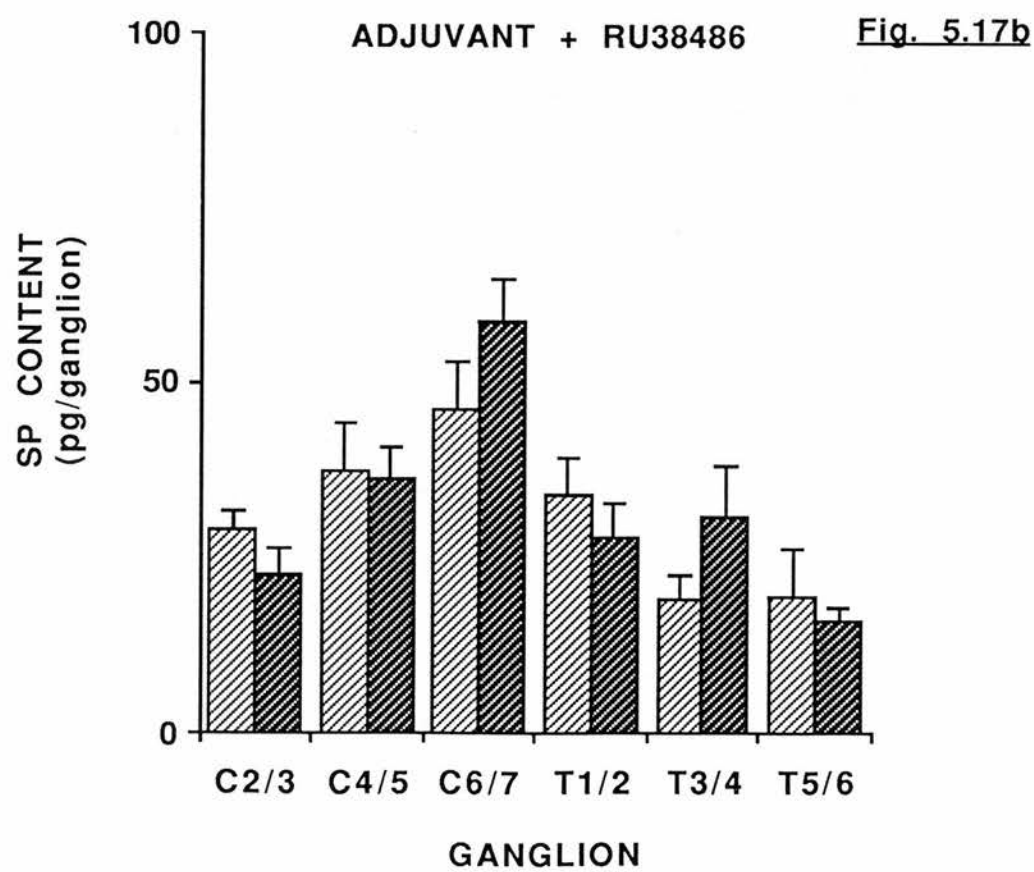
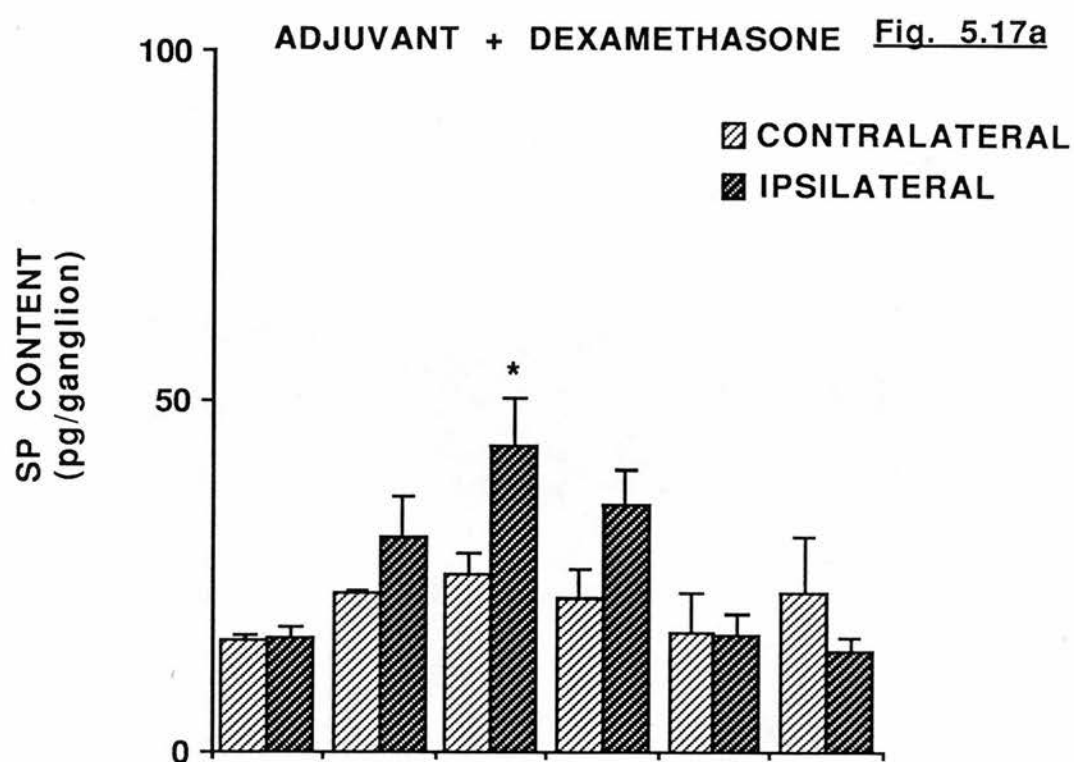
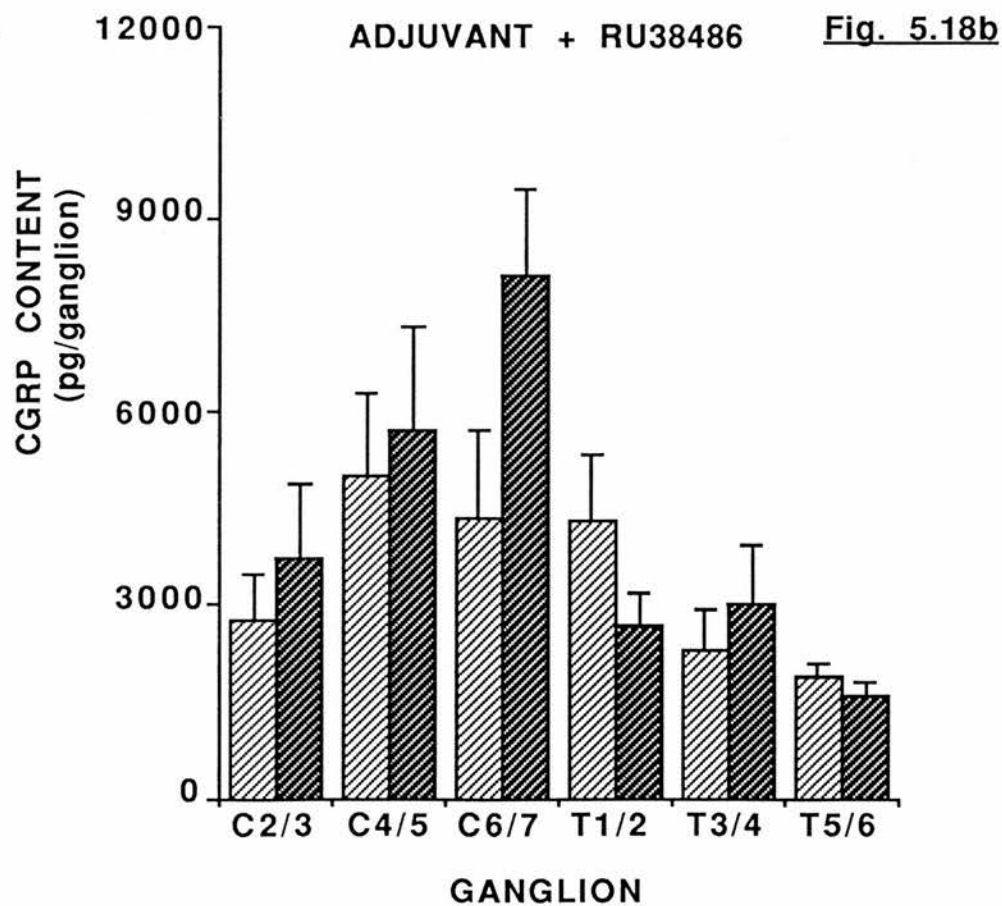
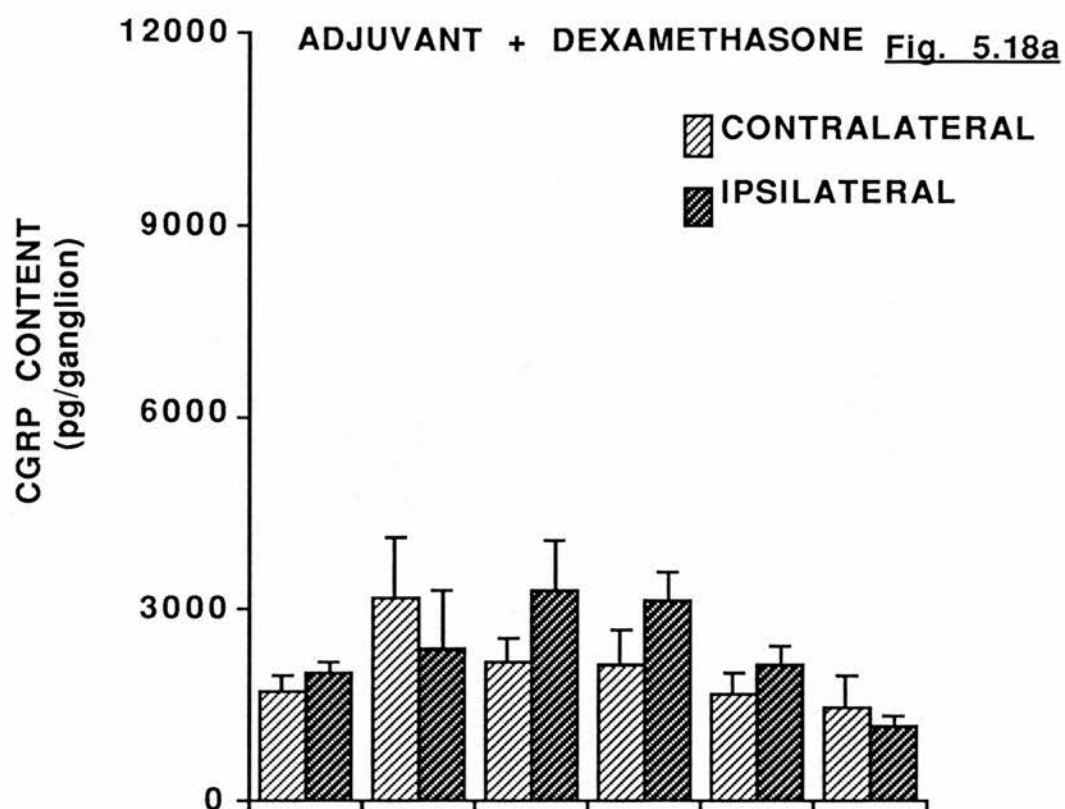


Figure 5.18 CGRP content (pg/ganglion) of *drg* in pairs C2/3, C4/5, C6/7, T1.2, T3.4 and T5/6 from ipsilateral and contralateral sides of animals injected with FCA and administered DEX (a) RU38486 (b) from days 10 to 20. Values are mean with S.E.M. to bar



content of ipsilateral *drg* with contralateral *drg* in DEX-treated animals revealed no significant difference between the two sides (ANOVA).

Effects of RU38486 on FCA-injected animals

The body weight of animals treated with RU38486 did not differ significantly (ANOVA) from that of the FCA control group at any point during the experiment (Fig. 5.9c). Compared with FCA treated control animals, RU38486 treatment did not significantly alter plasma B or ACTH, although ACTH did tend to be lower (Table 5.2, unpaired t-test). In both the FCA controls and the RU38486-treated animals, the circumference of the ipsilateral carpal joint was significantly ($p < 0.01$, ANOVA, Scheffe's F-test) greater than the contralateral from day 2 until the end of the experiment (Fig. 5.16). Treatment of FCA-injected animals with RU38486 from day 10 until the end of the experiment did not significantly alter the circumference of the ipsilateral carpal joint compared with FCA controls.

Compared with the SP content of *drg* from FCA-treated animals, RU38486 did not produce a significant change in any of the areas studied (ANOVA Fig. 5.17).

CGRP content of ipsilateral C6/7 *drg* of RU38486 treated animals was significantly ($p < 0.05$; ANOVA, Duncan's multiple range test) greater than that of FCA-treated controls (Fig. 5.18). Comparing *drg* from the two sides of RU38486-treated animals, no significant difference between ipsilateral and contralateral CGRP contents was apparent; although CGRP content of ipsilateral C6/7 *drg* tended to be greater than contralateral, this was not significant.

5.4 DISCUSSION

In the present study, I have investigated, using RIA, the neuropeptide content of *drg* following induction of monoarticular adjuvant arthritis in the left carpal joint of the rat. Injection of FCA or ICA resulted in an acute

inflammation of the ipsilateral joint over the first four days. In the carpal joint of the FCA injected animals, this initial acute inflammation was more severe and a period of secondary inflammation was apparent between 12 and 18 days after injection (Fig. 5.2a.). I have shown that 15 (Fig. 5.5a.) but not 20 days (Fig. 5.10a.) following induction of FCA evoked inflammation there was an increase in CGRP content of *drg* from level C6/7 of the ipsilateral side, but that no significant change in SP and SS content was detectable, although SP content did tend to be greater on the ipsilateral side. Whilst DEX suppressed the inflammation (Fig. 5.16a.), ADX exacerbated and extended the duration of the swelling of the affected joint to day 20 (Fig. 5.13a.), at which point the CGRP content of ipsilateral C6/7 *drg* (Fig. 5.14b.) was elevated. The CGRP content of the ipsilateral T1/2 *drg* of ADX animals was also significantly greater.

The evidence from other studies implicating neuropeptides in the pathology of adjuvant arthritis has been discussed in the Introduction. Weihe and colleagues (1988) described increased immunostaining for SP and CGRP in the dorsal horn and in peripheral inflamed tissues a proliferation of immunoreactive fibres was noted. Release of CGRP into the spinal cord of adjuvant arthritic rats inoculated in both hind paws, is significantly enhanced compared with controls (Nanayama et al., 1989) and CGRP content of lumbosacral *drg* of arthritic rats is also elevated (Kuraishi et al., 1989). Consequently, my own finding of increased CGRP in ipsilateral *drg* at C6/7 is in agreement with previous findings implicating CGRP in the inflammatory process and it may be that the increased content reflects elevated biosynthesis of CGRP to supply the reported proliferation of CGRPergic peripheral terminals and increased release of CGRP in the dorsal horn.

SP has also been implicated in the pathology of adjuvant arthritis from a number of studies, including that of Weihe (1988) described above.

Oku et al. (1987) reported that spontaneous release of SP into the spinal cord was enhanced in polyarthritic rats over controls and passive movement of inflamed joints, likely to cause pain, potentiated spontaneous release of SP. The suggestion that SP is directly involved in the transmission to the dorsal horn of pain produced by the movement is supported by the finding that in control animals, no increase in SP release was apparent following passive movement. Supporting the role of SP in inflammation, it has been shown that in the adjuvant rat model, severely arthritic joints (ankles) have a more dense SPergic innervation than more mildly arthritic joints (knees) (Levine et al., 1984). In addition, infusion of SP agonists or SP antagonists into the inflamed joint respectively potentiated and ameliorated the inflammation. In my own experiments I found a moderate but not significant increase in the SP content of *drg*. Lembeck and colleagues (1981) have reported a similar moderate, but not significant, rise in SP content of *drg* following induction of adjuvant arthritis, and Weihe et al. (1988) suggested that the changes in CGRP staining in the periphery and *drg* were more pronounced than those for SP. This does not, however, preclude a role of SP in adjuvant arthritis in peripheral tissues or the dorsal horn. It is possible that whilst the storage of SP is not enhanced in *drg*, the synthesis may be increased to supply the extra peptide requirement suggested by the results described above.

Recently, in contrast to mine and others results, Satoh (1990) and Ohno (1990) have reported increased SP, CGRP and SS content of *drg* in adjuvant and carageenin models of inflammation. It is possible that the model of arthritis used in this study produces a less severe inflammation of the joint, that does not involve SP or SS. Kuraishi et al. (1989) reported an increase in paw circumference of approximately 100% in contrast to the 50% increase found in this study. In that study, a higher dose of adjuvant was used to induce arthritis, and it is possible that in the less severe, low dose

model used here, the inflammation is not sufficiently pronounced to produce changes in SP content of *drg*. Interestingly, Kuraishi and colleagues (1989), using the same methodology as Ohno (1990), report a reduction in the withdrawal threshold of arthritic rats in the Randall-Silleto test. In the current study, while the adjuvant arthritis produce a relatively mild inflammation and erythema, the lesion did not appear painful, as the animals did not lose weight compared with untreated controls, groomed normally, and bore weight on the affected limb. Thus, it may be that SP is only important in painful inflammatory conditions. This suggests that CGRP is involved in mild inflammatory processes and SP is involved only in more severe, possibly painful inflammatory processes.

Whilst the origin of the sensory innervation of the carpal joint in the rat is not known, the changes in neuropeptides following localised, unilateral inflammation of this joint were restricted to ipsilateral C6/7 *drg*. The specificity of these changes suggests that principally C6/7 (and possibly T1/2) *drg* innervate the area of inflammation, and that changes in neuropeptide contents are due to local inflammatory processes and do not arise from systemic consequences of adjuvant arthritis. The sensory innervation of the carpal joint might be established by using one of the many retrogradely transported markers, such as true blue. Following intra-articular injection, this label is taken up by nerve terminals and retrogradely transported to the cell bodies. It is easily visualised in innervating *drg* using fluorescence microscopy.

My finding that ADX prolonged the inflammation and was associated with elevated CGRP content of ipsilateral C6/7 and T1/2 *drg*, and DEX suppressed the inflammation affirms the clinical use of glucocorticoids in the treatment of inflammatory disorders. The mechanisms by which glucocorticoids produce their anti-inflammatory activity have been discussed in the introduction to this Thesis (Chapter 1) and were shown to be complex,

varied and poorly understood. The results of my own study infer that a component of glucocorticoid anti-inflammatory activity might be to limit the availability of CGRP, a potent vasodilator (Brain et al., 1985) at peripheral terminals of primary afferents. Capsaicin destroys primarily C afferent fibres including SP and CGRP containing nerves and it has been shown that neonatal capsaicin treatments attenuates the oedema occurring in adjuvant arthritis (Levine et al, 1986). These findings support my contention that reducing the availability of neuropeptide in the periphery is anti-inflammatory, and my findings in Chapter 3 that glucocorticoids down-regulate CGRP content of *drg* supports the suggestion that this may be a part of their anti-inflammatory actions.

It should, however, be noted that the time course of the adjuvant evoked inflammation in the current series of experiments was variable. In the experiment investigating the role of glucocorticoids (Experiment 3), there was no significant difference in CGRP content of ipsilateral C6/7 *drg* compared with that of contralateral C6/7 *drg* of the FCA control group, in contrast to Experiment 2. Furthermore, in these animals, the inflammation was receding at the time the neuropeptide contents were determined. This does not alter the interpretation of the effects of ADX, which clearly exacerbated the inflammation and increased ipsilateral CGRP content. However, whilst DEX treatment equally clearly reduced the inflammation, it cannot be determined from the present results if this was also associated with a reduction in CGRP content of ipsilateral C6/7 *drg*. In future experiments, this question may be addressed if animals are killed at the time when the inflammation in FCA control animals is at a peak.

In contrast to the effects of ADX to prolong inflammation and elevate ipsilateral C6/7 *drg* CGRP content, RU38486, the Type II corticosteroid receptor antagonist, did not change the progress of the inflammation compared with FCA-injected controls. Furthermore, whilst the ipsilateral

C6/7 CGRP content of RU38486-treated animals tended to be greater than that of the contralateral, this was not significant. It is likely that the dose of RU38486 used in this study was insufficient to block the action of endogenous glucocorticoids. Evidence for this comes from the finding that whilst following ADX, plasma ACTH rose over 4-fold, plasma ACTH of RU38486-administered animals was slightly lower than FCA-treated controls. It is suggested that at the doses used in this study, RU38486 produced only incomplete antagonism of the anti-inflammatory actions of endogenous glucocorticoids.

The present results give no indication as to the mechanisms underlying the changes in CGRP content of *drg* in adjuvant arthritis. As discussed in the introduction to this thesis (Chapter 1), CGRP is contained in a large proportion of the cells of the *drg*, in both the large light and small dark populations. Whilst Weihe et al. (1988) reported that immunostaining for CGRP and SP was enhanced in cells of the *drg*, and there was no expansion of the immunoreactive populations, they did not report if this was in the total population, or a subset thereof. Given the effect of capsaicin, which destroys primarily C-fibres (principally, small dark cells), in reducing oedema in adjuvant arthritic rats (Levine et al., 1986), it would be of interest to establish if solely this population of CGRP containing cells showed enhanced immunoreactivity. This might be determined using immunohistochemistry, which would also allow investigation of the response in those cells co-localising SP and CGRP. The use of consecutive sections stained for SP or CGRP immunoreactivity, or double labelling techniques, would be useful in such a study.

In the *drg*, neuropeptides are synthesised in the soma and transported in vesicles to the peripheral or central terminals, although some peptide is degraded in the soma. Consequently, an increase in peptide content of the *drg* may reflect reduced axoplasmic transport from the *drg*,

increased synthetic rate, or reduced degradation rate. Whilst the evidence of other authors (Weihe et al., 1988) would suggest that the transport of CGRP and SP must be elevated to supply the expanded ramifications of the peripheral and central terminals, this might be established in studies using ligation of peripheral nerve (MacLean 1987; MacLean & Lewis 1984; see Introduction to Chapter 3).

Given the chronic nature of adjuvant evoked inflammation, it is possible that the CGRP content was increased through elevated gene transcription, mRNA translation or reduced mRNA degradation. Whilst this is of interest in itself, it may also be of relevance in considering the actions of glucocorticoids as they act principally, if not exclusively, on the genome to influence transcription (see Chapter 1). This question might be examined by use of a Northern blot or nuclease protection assay for CGRP mRNA. Both techniques would also indicate if α or β CGRP or both species was involved in the pathology of adjuvant inflammation. *In situ* hybridisation, using probes specific for either α or β CGRP mRNA would also be useful in investigating the cell population involved in the response to adjuvant inoculation.

In conclusion, I have demonstrated, using an adjuvant model of rheumatoid arthritis, increased CGRP content of *drg* but not of SP and SS during inflammatory processes. I have further shown that DEX reduced the inflammation whilst ADX prolonged the duration of inflammation and this was associated with an increase in the CGRP content of C6/7 and T1/2 *drg*. I speculate that part of the anti-inflammatory action of glucocorticoids might be to regulate the availability of CGRP at primary afferent terminals in the periphery.

CHAPTER 6

GLUCOCORTICOID REGULATION OF SP AND CGRP *IN VITRO*:

DISSOCIATED PRIMARY CULTURE OF ADULT RAT *DRG*

6.1 INTRODUCTION

Harrison (1907) was the first to culture nervous tissue; a fragment of frog neural tube placed in a drop of clotted lymph on a coverslip which was inverted and sealed over a depression slide. With suitable aseptic precautions, cultures could be maintained for up to four weeks and the development of nerves observed. For culture of avian and mammalian tissue, the lymph was replaced by plasma and embryo extract provided the nourishment required which, in the frog, is contained within the embryonic cells themselves. Further developments included culture of sympathetic ganglia explants on chick embryo intestinal wall placed in saline solutions and the use of cerebrospinal fluid as a medium for mammalian cerebral cortex. In general, however, cultured avian or mammalian tissue underwent a characteristic granular degeneration within one or two weeks (see Murray, 1965).

From these initial experiments, technical developments in the composition of the medium and nature of supplements, the substrate to which cultures attached and the vessels in which they were grown improved the viability of cells and produced cultures which more closely resembled tissues *in vivo*. Noteworthy amongst these developments was the addition of foetal or placental serum to media and the addition of glucose supplement to the medium. Reduction of toxic trace elements in reagents used to produce media, and use of collagen-coated coverslips to allow cells to adhere, rather than be held stationary in plasma clots further enhanced survival of neuronal cultures (see Murray, 1965; Nelson, 1975).

Whilst initial studies were concerned with tissue explants held in plasma clots, Nakai (1956) produced cultures of dissociated chick embryo *drg* by digestion with proteinase followed by gentle trituration. The resulting cell suspension, contained in a balanced salt solution, was added to an equal amount of chick embryo extract alone or supplemented with human

placental serum or bovine serum. This mixture was then clotted using heparinised cockerel plasma and placed in a Maximow slide preparation. Incubated at 37°C, the cultures could be maintained for up to 19 days. A number of improvements in this technique followed; the development of chemically defined media, sufficient to maintain neurons in culture when supplemented with foetal calf serum was of particular significance (Scott et al., 1969). The use of collagen substrata and a rigorous study of the digestion agents (Banks et al., 1970) also enhanced the survival of dissociated cultures.

Whilst the vast majority of early studies used embryonic or early post natal tissue for culture, initially there were relatively few reports of successful culture of adult neurons. Adult human sympathetic ganglia (Murray & Stout, 1974,1975) adult rat sympathetic ganglia (Silberstein et al., 1971, 1972), adult frog *drg* (Padjen et al., 1975) and adult frog sympathetic ganglia (Hill & Burnstock, 1975) were notable exceptions but it was not until 1977 that the first report of dissociated culture of mammalian (mouse) adult *drg* appeared (Scott, 1977). In this technique, *drg* were 'softened' in 0.25% collagenase before being split open with microforceps and triturated in a firepolished pipette. The cells were cultured on collagen-coated coverslips in CMRL-1415 medium supplemented with 10% foetal calf serum. A variety of media have since been used to maintain dissociated cultures of *drg* but the medium used in this study is based on that of Lindsay (1988).

In these early experiments, no attempt was made to reduce the numbers of non-neuronal cells. However, non neuronal cells are mitotically active and consequently, with time tend to multiply and cover the culture plate, compromising the survival of neurons. Furthermore, the presence of non-neuronal cells complicates interpretation of experiments utilizing cultures of *drg* neurons as it is now known that such cells produce growth factors, including NGF which are important in supporting the survival and/or

stimulating outgrowth of neurites from both neonatal and adult *drg* neurons (Varon et al., 1974; Wrathall, 1982; Roufa et al., 1983). Interestingly, it has also been claimed that non-neuronal cells regulate SS content of cultured sensory neurons (Mudge, 1981). Initially, methods for purifying neurons from non-neuronal cells in *drg* preparations were based on that employed in the purification of chick embryo cortical cells (Varon et al., 1969).

Suspension of *drg* were plated for approximately three hours onto layers of glass beads, to which the non-neuronal cells preferentially adhered. The supernatant containing purified neurons was then removed and plated on to culture dishes. With this method, an increase in the percentage of neurons from 40% to 80% was achieved (Okun, 1972). Another technique relied on the differential sedimentation of the two cell types under centrifugation, in which neurons were gently pelleted leaving the smaller non-neuronal cells and connective tissue debris in the supernatant (Lodin et al., 1970; Miller et al., 1970). Passing *drg* suspensions through Percoll gradients also proved useful (Goldenberg & De Boni, 1983). In the current study, non-neuronal cell numbers were reduced using a method based on that described by Lindsay (1988). This technique relies on a combination of cleaning *drg* of connective tissue to reduce initial non-neuronal cell contamination, preferential adherence of non-neuronal cells to a polyornithine substrate (McCarthy & Partlow, 1976) and differential sedimentation of the two cell types.

Whilst the pre-plating procedure reduces the non-neuronal cell population a certain proportion of such cells remain following plating onto laminin. Given that such cells are mitotically active and tend to proliferate in culture it is desirable to inhibit their multiplication by inclusion in the medium of a mitotic inhibitor. Typically, in previous studies the chemotherapeutic agent cytosine arabinoside (Ara-C), a structural analogue of 2'-deoxycytidine was included for such a purpose (Lindsay, 1988; Lindsay et

al., 1989). Despite extensive study, the precise mechanism of action of Ara-C is not clear (Calebresi & Parks, 1985), although it has been found to inhibit DNA strand elongation following phosphorylation to an active intermediate that substitutes for dCTP (Kufe & Major, 1982). However, when used clinically in the treatment of leukaemia patients occasionally suffer from a cerebellar toxicity syndrome in which Purkinje cells die, despite the fact that such cells are not mitotically active (Sylvester et al., 1987; Winkelman & Hines, 1983).

Such observations have led to a re-evaluation of the use of Ara-C as a mitotic inhibitor in the culture of neuronal cells. Wallace and Johnson (1989) have recently reported that Ara-C produces a dose dependent inhibition of potassium and insulin stimulated survival of postmitotic (E8) chick ciliary ganglion neurons and the NGF stimulated survival of postmitotic chick drg neurons. This cytotoxic effect of Ara-C was blocked by 2'-deoxycytidine, but this factor did not of itself enhance survival. Furthermore, it has been demonstrated that the morphological and temporal characteristics of neuronal death following NGF deprivation or Ara-C treatment on post mitotic rat sympathetic neurons were indistinguishable (Martin et al., 1990). Other means of producing neuronal cell death such as ultraviolet irradiation and ricin toxin did not share these characteristics. Thus, there is now some doubt as to the wisdom of using Ara-C as an antimitotic agent in the preparation and maintenance of non-neuronal cell free cultures. On consideration of the evidence indicating the dubious suitability of Ara-C as an antimitotic agent I used an alternative method for the control of non-neuronal cell proliferation.

Kao & Prokop (1977) have shown that a number of proline analogues are incorporated into protein during procollagen biosynthesis in fibroblasts. Furthermore, they demonstrated that culture of fibroblasts in the presence of cis-4-hydroxyproline (cis 4-OH) markedly reduced their growth rate. Whilst

the mechanism of action of this agent is not clear it is thought that it prevents deposition of triple helical collagen at the cell surface, possibly inhibiting adhesion of cells to the substratum. Thus inclusion of *cis* 4-OH proline in the culture medium would appear to offer an expeditious, non-toxic method of controlling fibroblast growth in cultures of *drg*. Observations of my own cultures suggests that it also prevents generalised proliferation of non-neuronal cells although this has not been quantified.

The major advantage of primary cultures of adult *drg* over embryonic or post natal cultures is that NGF or other growth factors are not required for cell survival. Lindsay (1988) has demonstrated using cultures of single cells, in the absence of non-neuronal cells, that *drg* neurons can survive in fully defined medium (i.e., F.14 supplemented with insulin, transferrin, selenium, progesterone and putrescine) for up to seven days in culture. Whilst NGF and BDNF were not required for neuronal survival, they did enhance neurite formation and NGF has been found to regulate SP and CGRP gene expression and peptide content (Lindsay et al., 1989; Lindsay & Harmar, 1989). The SS content of *drg* in culture rapidly falls to low levels and is not maintained in the presence of NGF or other growth factors (A. J. Harmar personal communication) and thus it is not currently practical to study regulation of *drg* SS in the the dissociated culture system. However, the adult rat *drg* primary culture system offers the opportunity to examine regulation of SP and CGRP in a fully defined medium.

In the current study, I have developed primary culture of adult rat *drg* and used this system to investigate adrenal steroid regulation of SP and CGRP content of *drg*. Previously, in collaboration, I have demonstrated, using *in situ* hybridisation histochemistry, the presence of mRNA for Type I and Type II corticosteroid receptors in *drg* (J. Seckl et al, unpublished observations). Furthermore, I have demonstrated that SP and CGRP content of *drg* increased following ADX and that this was reversed by

administration of glucocorticoids. However, there was no indication from these *in vivo* experiments of the site of action of glucocorticoids; directly at the *drg* or by some other, secondary intermediate. One way of resolving this question is to examine the role of glucocorticoids in regulation of SP and CGRP in purified culture of neurons.

6.2 METHODS

Between four and six Cob Wistar rats (male, 200g) were killed by decapitation, *drg* dissected free and dissociated cultures of *drg* enriched for neurons prepared as described previously (Chapter 2). Cells were plated onto 35mm four well plates coated with laminin at a density of 3000-5000 cells/well (constant within each experiment). Medium containing antibiotics was supplemented with either 4% USG or the defined serum supplement ITS+ containing insulin, transferrin, selenium and fatty acids; cis 4-OH proline (200µg/ml) was added to inhibit non-neuronal cell proliferation. Medium was changed on day 1 and every 2 days thereafter and samples were extracted from each well separately in 80µl 2M acetic acid and stored at -70°C before RIA for SP or CGRP.

A number of experiments were performed to investigate the action of corticosteroids on neuropeptide content of *drg*. Experiments were carried out over 3 or 5 days and samples were extracted on days 0, 1, 3 and 5. The effect of forskolin (10µM), B (100µg/ml), DEX (10µM) and NGF (25ng/ml) on SP and CGRP content of *drg* in medium supplemented with either ITS+ or USG was initially studied. Subsequently, the effect of dexamethasone and corticosterone on NGF induced increase in SP and CGRP content of *drg* was examined.

6.3 RESULTS

6.3.1 Effect of NGF

In the absence of NGF, SP and CGRP contents of *drg* grown in medium containing USG or ITS+ fell over 5 days from the time of plating. NGF administration maintained the SP and CGRP contents of *drg* in media supplemented with USG or ITS+ compared with untreated controls over the same period (Fig. 6.1a,b).

6.3.2 Effect of forskolin

As in previous studies, SP and CGRP contents of *drg* decreased over the 3 days from the time of plating when grown in either USG or ITS. Forskolin administration over the same period produced a significant increase in both SP (Fig. 6.2a) and CGRP (Fig. 6.2b) content of *drg* grown in medium supplemented with either USG or ITS when compared with the untreated controls.

6.3.3 Effect of Dex and B

In the absence of NGF, SP and CGRP content of cultures fell rapidly from the time of plating; concomitant administration of DEX (Fig. 6.3a,b) or B (Fig. 6.4a,b) did not influence this reduction. Fig. 6.5a,b shows the effect of DEX and B on SP and CGRP content of *drg* grown in ITS+ supplemented medium in the presence of NGF. SP content fell slightly from the time of plating, but concomitant administration of DEX or B significantly reduced the SP content over the same period. CGRP content increased from the time of plating, and administration of DEX or B significantly reduced CGRP content.

6.4 DISCUSSION

As found by previous authors, I have shown that NGF is required in order to maintain SP and CGRP content of *drg* in culture. Administration of

Figure 6.1 The effect of NGF (25ng/ml) on SP (a) and CGRP (b) content (pg/well) of neuron enriched cultures of *drg* grown in medium supplemented with ITS+ or USG (4%).

Values are mean with S.E.M. to bar (n = 8)

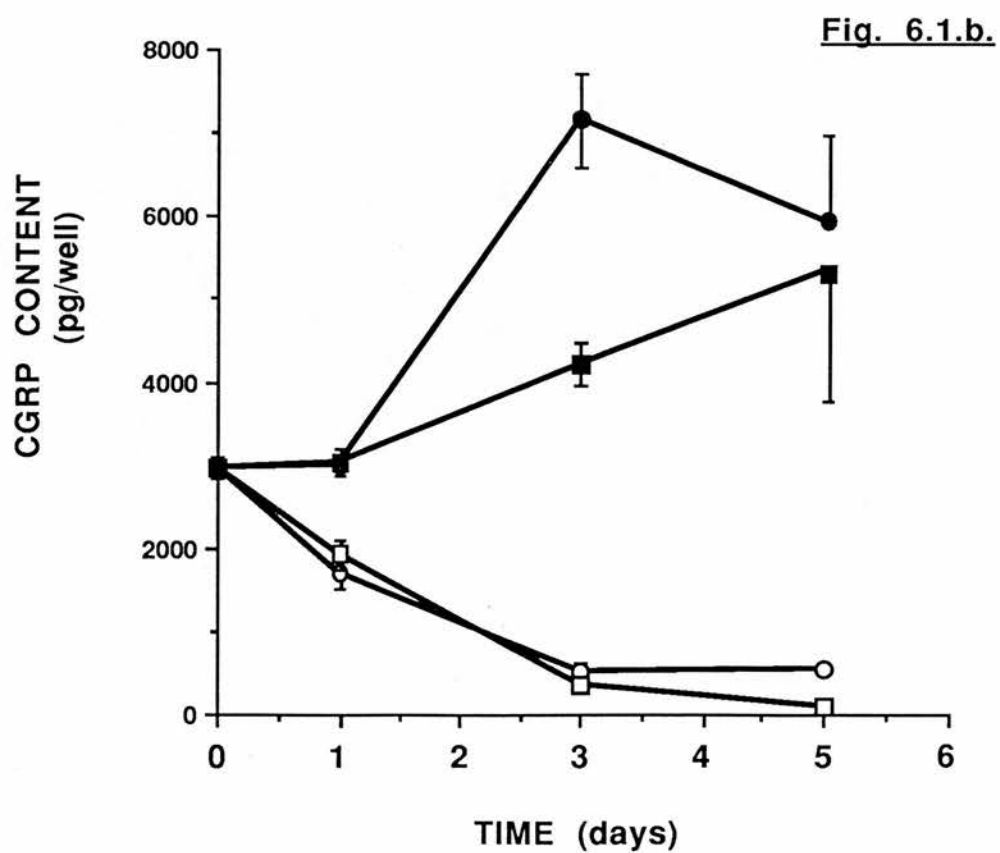
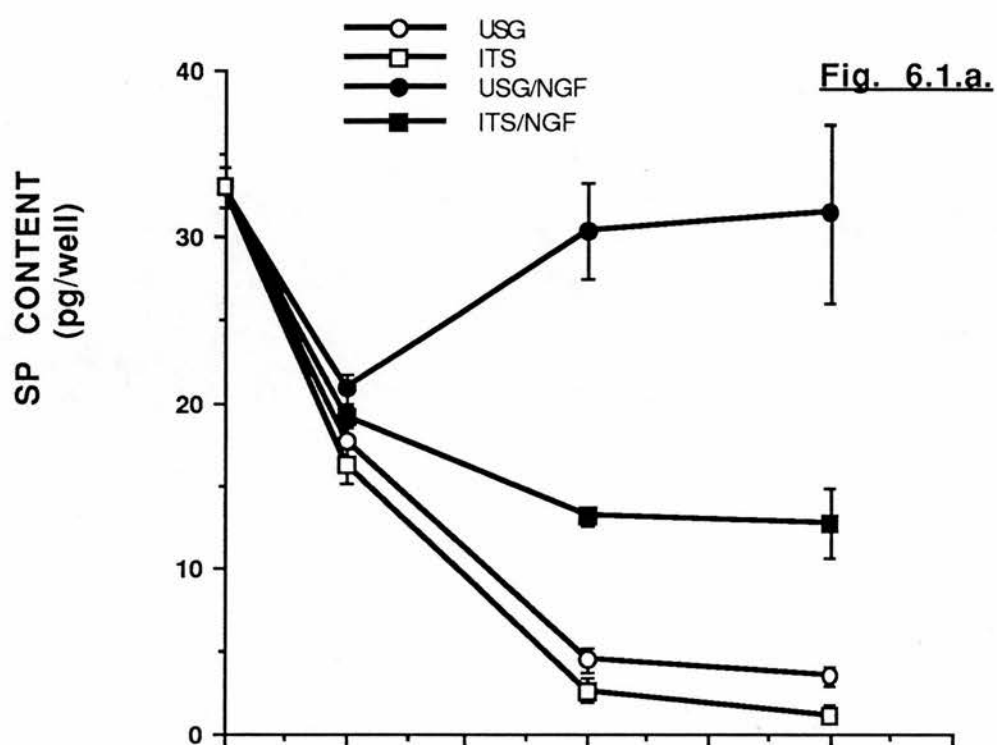


Figure 6.2

The effect of forskolin (FK; 10 μ M) on SP (a) and CGRP (b) content (pg/well) of neuron enriched cultures of *drg* grown in medium supplemented with ITS+ or USG (4%).

Values are mean with S.E.M. to bar (n = 8)

Fig. 6.2.a.

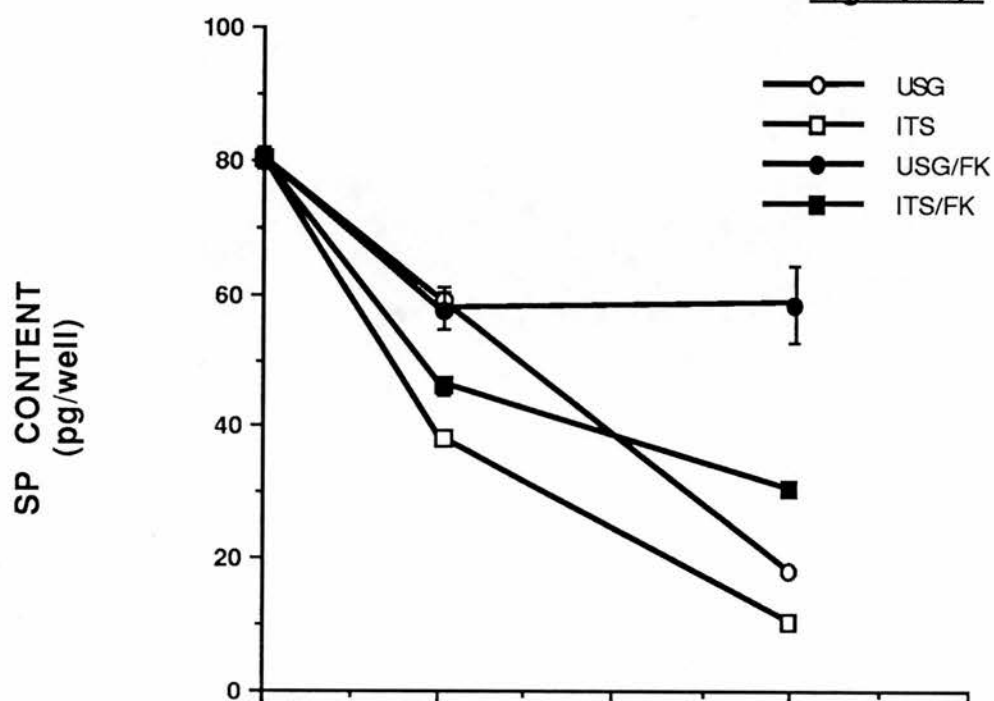


Fig. 6.2.b.

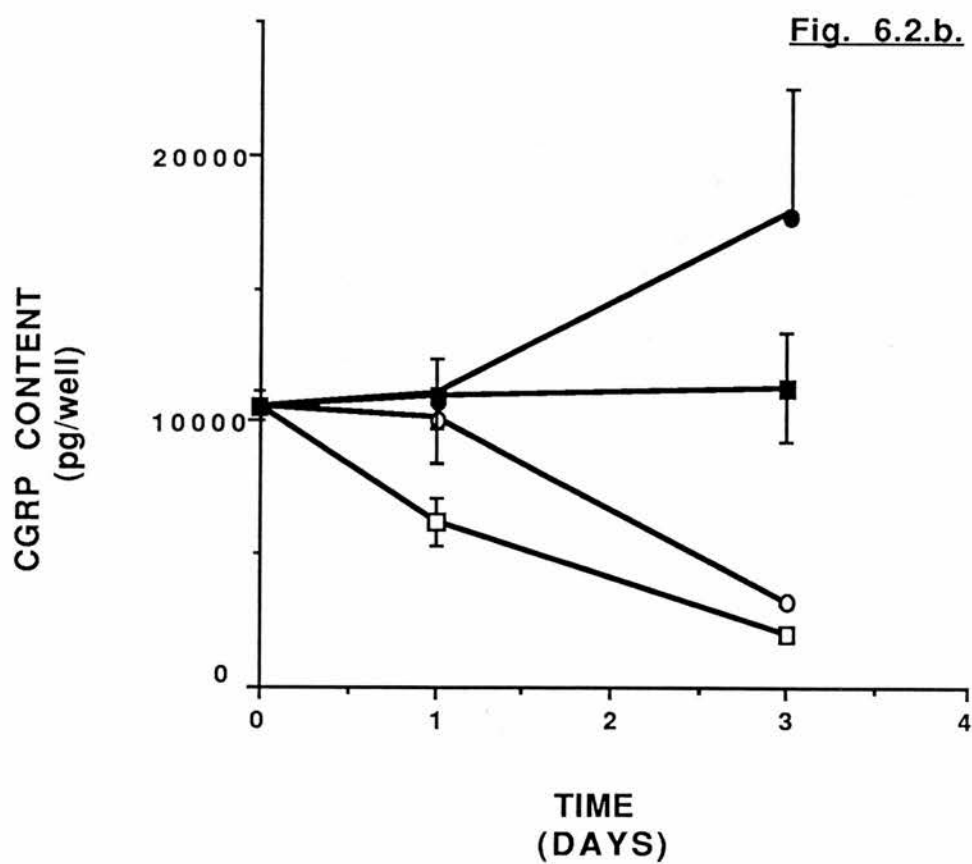


Figure 6.3 The effect of DEX (10 μ M) on the SP (a) and CGRP (b) content (pg/well) of neuron enriched cultures of *drg* grown in medium containing ITS+ or USG (4%). Values are mean with S.E.M. to bar (n = 8)

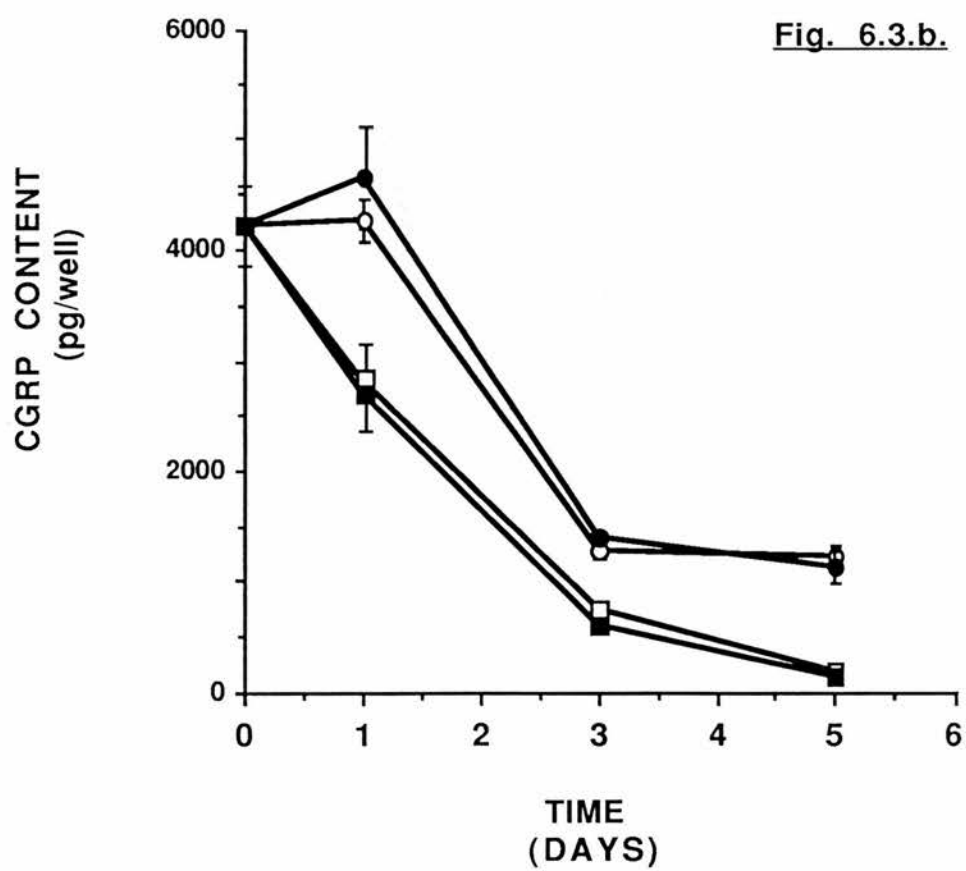
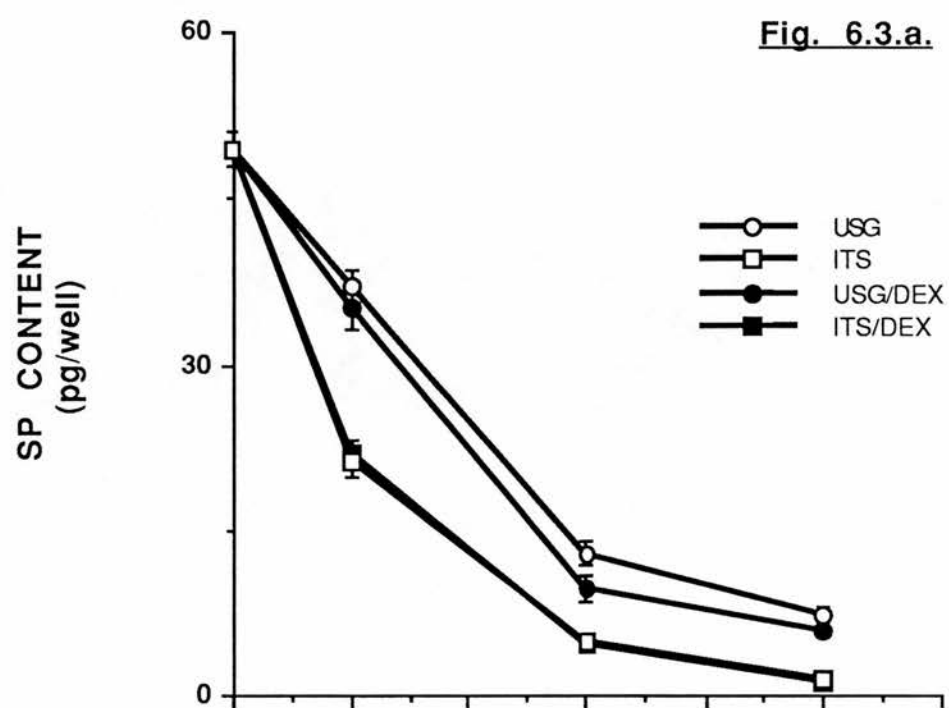


Figure 6.4 The effect of B (100 μ g/ml) on SP (a) and CGRP (b) content (pg/well) of neuron enriched cultures of *drg* grown in medium containing ITS+ or USG (4%). Values are mean with S.E.M. to bar (n = 8)

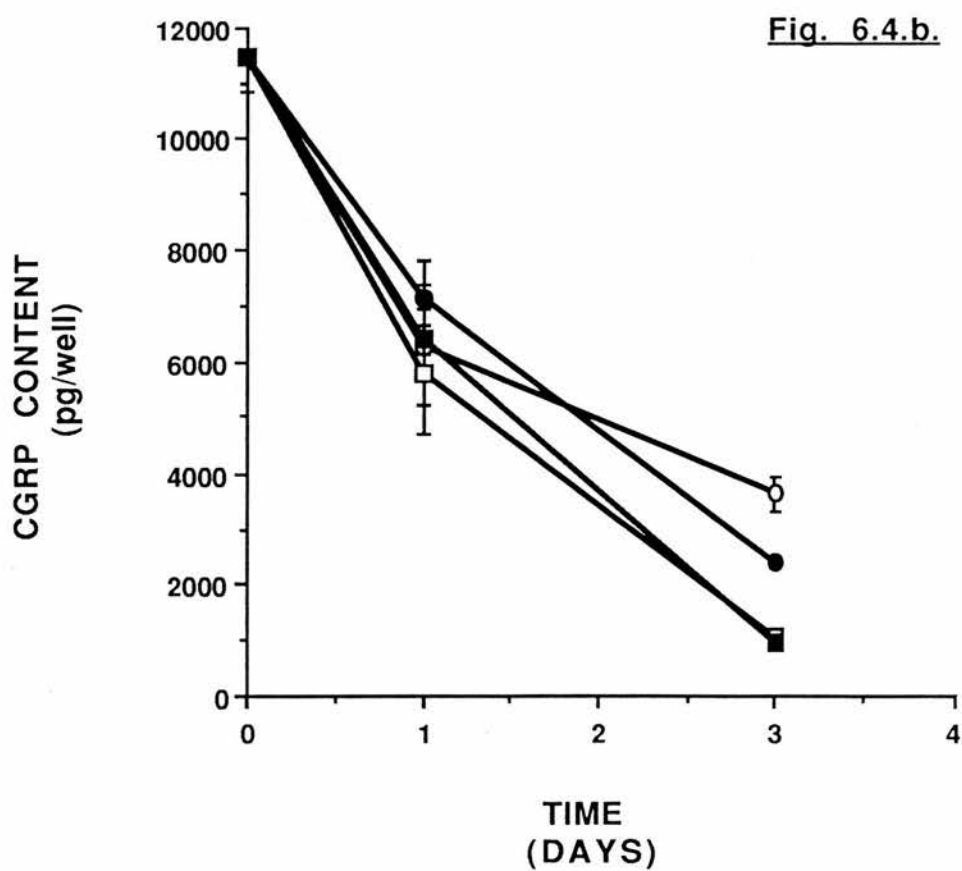
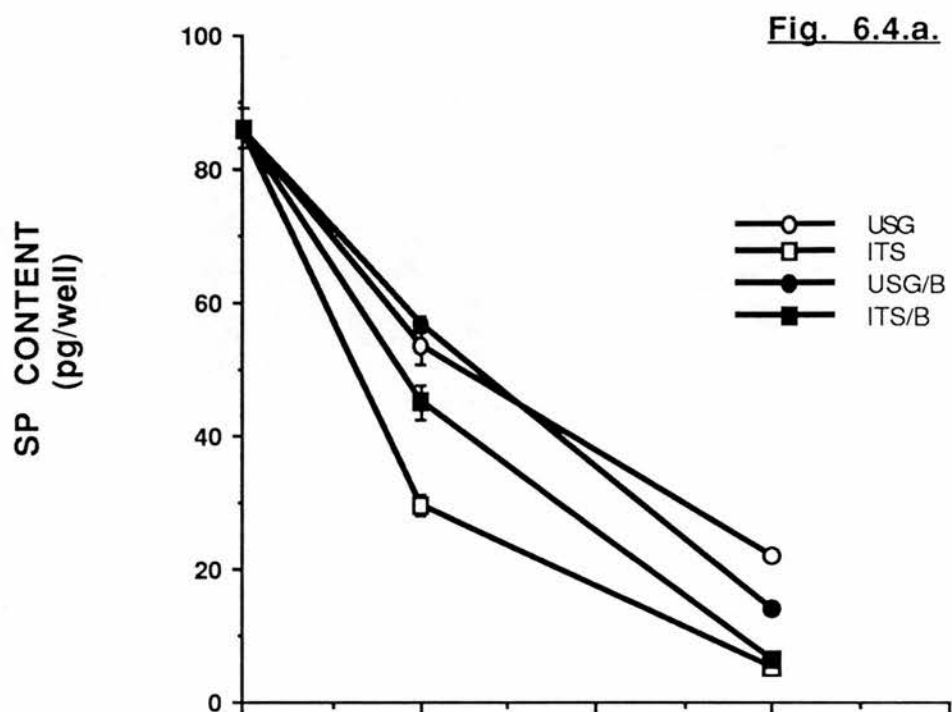


Figure 6.5

The effect of DEX (10 μ M) or B (100 μ g/ml) on SP (a) and CGRP (b) content (pg/well) of neuron enriched cultures of *drg* grown in medium supplemented with ITS+ and 25ng/ml NGF (n = 8)
(*p < 0.05, *** p < 0.001, c.f. NGF only; unpaired t-test)

Fig. 6.5.a.

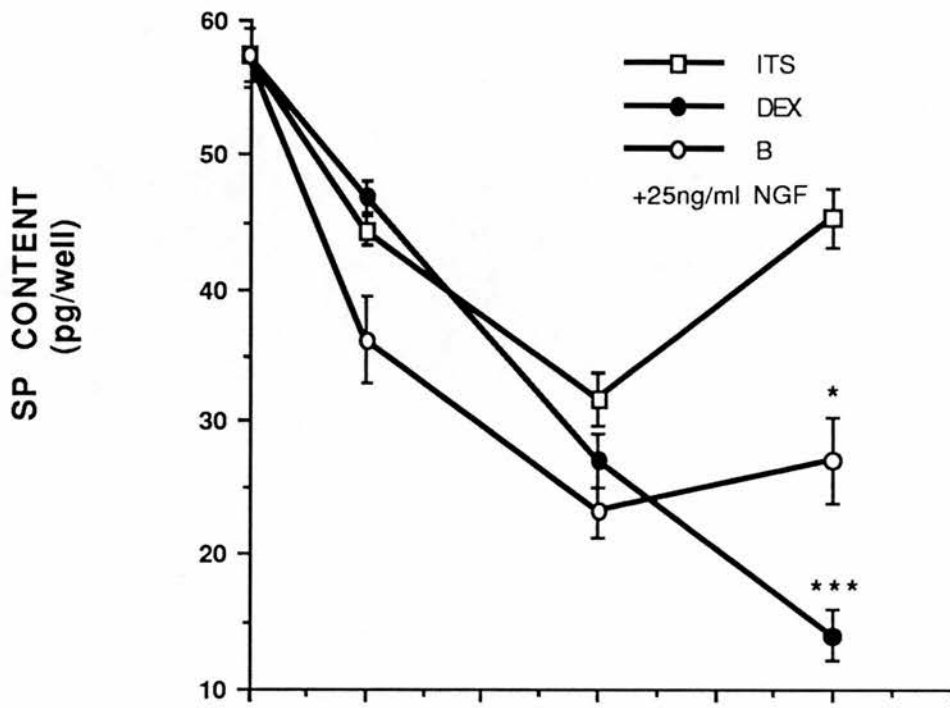
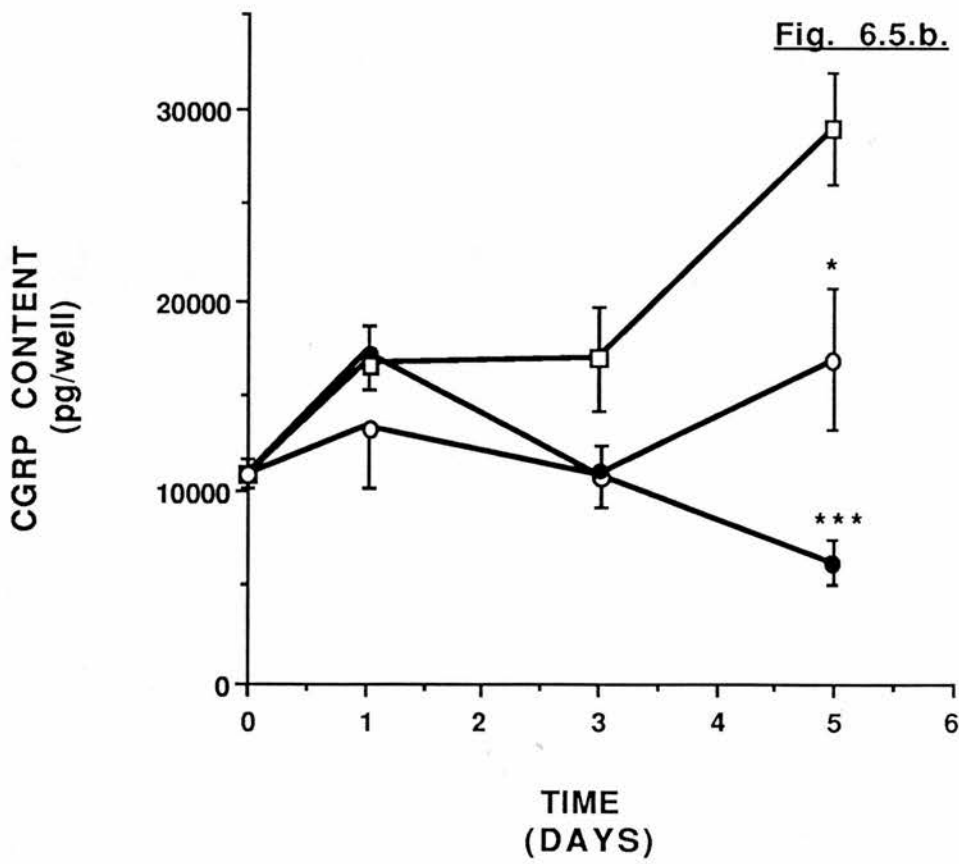


Fig. 6.5.b.



DEX or B reduced NGF maintained SP and CGRP content, but of themselves did not appear to influence the SP and CGRP content of cultures. These results suggest that adrenal steroids regulate SP and CGRP content of *drg* directly, possibly by antagonising the action of NGF. Furthermore, I have found that forskolin, which activates adenylate cyclase leading to increased c-AMP production, stimulated an increase in both SP and CGRP content of cultured *drg*.

The finding that DEX and B reduced the SP and CGRP contents of cultures grown in the presence of NGF is consistent with my observations *in vivo*. Following ADX, SP and CGRP contents of *drg* were increased, and this was prevented by administration of DEX or B (see Chapter 3). In other tissues, glucocorticoids has also been shown to regulate neuropeptides. DEX reduced SP content of rat neonatal superior cervical ganglia in dissociated culture in the presence of NGF (Kessler et al., 1983). NGF increased both SP and CGRP content of cultured neonatal rat vagal sensory neurons, whilst B reduced SP but not CGRP content independently of NGF (MacLean et al., 1989). In the clonal rat C-cell derived 44-2C cell line, DEX significantly reduced the levels of CGRP-specific mRNA (and calcitonin peptide and mRNA; Zeytin et al., 1987).

The PC12 cell line, derived from a rat pheochromocytoma responds to both NGF and glucocorticoids, and synthesises a number of neuropeptides including neuropeptide Y (NPY) and neurotensin (NT). The NPY content of PC12 cells grown in the presence of NGF increases, and this is inhibited by concomitant administration of DEX (Sabol & Higuchi, 1990). In contrast, intracellular content and synthesis of NT is substantially and synergistically increased by the combined action of NGF and DEX (Tischler et al., 1982, 1986). Further studies have demonstrated that these agents act to increase mRNA encoding NT/neuromedin (Dobner et al., 1988).

PC12 cells grown in the presence of glucocorticoids round up and differentiate to a cell type with a phenotype resembling an endocrine adrenal chromaffin cell, and cultures grown in the presence of NGF tend to stop dividing, flatten down on to the substratum and extend neurites. Similarly, in cultures of dissociated rat adrenal chromaffin cells, it is found that NGF stimulates fibre outgrowth, and this is abolished by glucocorticoids (Unsicker et al., 1978). Sympathetic neurons grown in dissociated culture elaborate neurites in response to NGF, and this is inhibited, but not completely abolished by administration of glucocorticoids. It is now considered that glucocorticoids influence differentiation of the sympathoadrenal lineage derived from neural crest precursors; thus, NGF promotes development of such cells into noradrenergic neurons and glucocorticoids stimulate the development of so-called 'SIF' (small intensely fluorescent) cells (Landis & Patterson, 1981). It appears that the apparently antagonistic link between the actions of NGF and glucocorticoids, whilst not immutable, is a commonly encountered phenomenon, both in neuropeptide regulation, and differentiation and development.

The glucocorticoid receptor contains a region, known as the DNA binding domain (domain C, see Fig. 1.4), within which is found two zinc-finger motifs. Such structures are thought to allow the ligand activated receptor to bind, as a dimer to DNA in the 5' regulatory regions of genes. Analysis of the sequence specificity of binding has allowed construction of a 15 mer base pair sequence which shows greatest specificity for binding of the glucocorticoid receptor, the so-called glucocorticoid response element (GRE(+)). It is thought that glucocorticoids produce their effects by binding to this sequence to activate gene transcription (see Chapter 1 for full description and references). Given that SP and CGRP content was reduced by glucocorticoids, it may be that this was due to a direct action on a GRE(-)

(see Chapter 1), the binding of glucocorticoids to which is thought to down-regulate gene expression.

Sequence analysis of 800bp of the 5' flanking region of the rat PPT gene and 1.8kb of 5' flanking sequence of the human calcitonin/ α -CGRP gene reveal no sequences with homology to the GRE(+) (Carter & Krause, 1990; Broad et al., 1989). Currently, no consensus sequence is available for the GRE(-) and thus it is not known if such a region is contained within the regulatory sequences of the SP or CGRP genes. Alternatively, it may be that they are contained in regions not yet sequenced or glucocorticoids may influence transcription by regulation of an unknown, trans-acting factor.

In the current study forskolin, an activator of adenylate cyclase was found to mimic the effects of NGF on SP and CGRP content. A number of other biochemical and transforming actions of NGF such as NGF induced phosphorylation (Halegoua & Patrick, 1980) and neurite formation (Schubert et al., 1978) can also be mimicked by c-AMP analogues; although qualitative differences are apparent in the effects of NGF and c-AMP agonists. These differences in the actions of the two agents, the findings that NGF does not activate adenylate cyclase (Race & Wagner, 1985) and c-AMP antagonists do not block the neurotrophic effects of NGF (Rydel & Greene, 1988) suggests that the actions of NGF may not be mediated solely or directly by a c-AMP second messenger. Furthermore and most convincingly it has been found that a PC12 clone, deficient in c-AMP dependent protein kinase is still NGF responsive (Rydel & Greene, 1988). Inositol phosphate second messengers and tyrosine phosphorylation have also been implicated in the action of NGF and it appears that NGF actions are probably mediated via more than one second messenger signal (see Cho et al., 1989; Levi & Alemà, 1991).

That forskolin mimicked the effects of NGF on SP and CGRP content, suggests that the two peptides may be regulated at the level of the genome

by the action of c-AMP second messenger on a c-AMP response element (CRE). A similar finding, that forskolin mimics the effect of NGF on SP and CGRP peptide content has been reported for rat neonatal vagal sensory neurons in culture (MacLean et al., 1989). In this study, it was further shown that forskolin also increased SP but not CGRP content of cultures in the presence of maximal doses of NGF, indicating that other mechanisms or ligands mediated by adenylate cyclase, may regulate SP content in this tissue.

Cote et al. (1990) coupled the first 146bp of the 5' regulatory region of the calcitonin/CGRP gene, to a human growth hormone reporter gene and transfected this into TT cells (derived from a medullary thyroid carcinoma). Studies demonstrated that expression of this construct was up-regulated by cAMP mimetics. In another medullary thyroid carcinoma cell line (MTC cells), secretion of calcitonin and expression of calcitonin mRNA was found to be up-regulated by forskolin (Haller-Brem et al., 1988). Sequence analysis of the regulatory regions of the human calcitonin/ α -CGRP gene has identified two potential CREs one with two inverted copies of the consensus sequence and one with a single copy of the consensus sequence (Broad et al., 1989). Whilst no studies are known of cAMP regulation of the tachykinin gene, sequence analysis has identified two regions with homology to the CRE consensus sequence (Carter & Krause, 1990). My findings suggest that in *drg*, forskolin may stimulate production of cAMP possibly leading to enhanced SP and CGRP gene transcription and synthesis, resulting in increased peptide content. Interestingly, glucocorticoid inhibition of the human glycoprotein hormone α subunit has been found to occur as a consequence of the close proximity of a GRE(+) and a CRE. Negative regulation of gene expression is thought to occur when glucocorticoid receptors bind to the DNA and impair binding of transcription factors acting through the CRE (Akerblom et al., 1988). Given the lack of GREs in the

regulatory regions of the CGRP and tachykinin genes, it is not thought likely that such a mechanism would account for my findings.

The SP and CGRP contents of NGF maintained cultures were reduced significantly by DEX and B, both of which were used at a high dose. DEX was more effective than B, suggesting this action could be mediated via Type II receptors but, from this single dose observation, it is not possible to state this conclusively. Previously, in collaboration with Dr. J.S. Seckl, I have demonstrated, using *in situ* hybridisation, the mRNA for both Type I and Type II receptors in the neurons of the *drg*. Thus, adrenal steroids may, through either receptor, regulate neuropeptides in the *drg*. Further studies utilising the recently developed Type II specific agonists and antagonists would provide conclusive evidence as to the nature of the corticosteroid effect. In further experiments, I would suggest that antagonism of DEX and B suppression of SP and CGRP contents by the Type II specific antagonist RU38486 and use of RU28362 (a Type II specific agonist) to mimic the actions of DEX and B should provide sufficient evidence as to the nature of the receptors mediating steroid inhibition.

The molecular mechanisms underlying the maintenance of SP and CGRP contents of *drg* neurons in dissociated culture have been investigated previously (Lindsay & Harmar, 1989). Following culture of dissociated neurons in the absence of NGF, a fall in SP and CGRP peptide was paralleled by a reduction in mRNA encoding the two peptides. It was further demonstrated that NGF stimulated an increase in SP and CGRP peptide, preceded by increased SP and CGRP mRNA content. It would be of interest to extend the results of my current study by determining if glucocorticoids reduce SP and CGRP peptide content by an action of the transcription of the respective genes. As discussed earlier, this is of particular interest, given the proposed mechanism of action of glucocorticoids, to influence gene transcription. Whilst the Northern blot

would allow determination of total mRNA for SP or CGRP, the production of a number of PPT mRNAs and the role of mRNA splicing in the regulation of CGRP mRNA expression suggests that a nuclease protection assay might be more informative. Such a technique, if the appropriate probes are available, allows assessment of changes in the splicing pattern of mRNA, in addition to changes in the total mRNA content.

Nuclease protection assays provide information regarding splicing but do not identify regulatory sequences in the 5' non-coding region of the gene. Such sequences can be identified using constructs comprising the 5' regulatory regions of genes and a reporter gene such as chloramphenicol acetyltransferase (CAT; Seed & Sheen, 1988). These constructs can be transfected into an appropriate cell line, site directed mutagenesis used to delete or reconstruct specific sequences of the regulatory region to determine their importance in control of reporter gene expression by particular ligands. Preliminary studies of this nature, using PC12 cells transfected with 1.8kbp of bovine PPT gene regulatory sequences (1.9kbp) indicate that glucocorticoids do not influence gene transcription (C. Gilchrist, personal communication). However, it may be that the sequences responsible are not contained in the fragment transfected, or the PC12 cell line may not be an appropriate model in which to study this phenomenon. PC12 cells synthesise SP at very low levels, and it may be that they are deficient in some unknown protein necessary for expression of the PPT gene. It is possible that the recently developed ND cell lines (produced by fusion of neonatal-rat *drg* and N18Tg2 mouse neuroblastoma cells) might be more appropriate for such work. These cells are found to contain relatively large amounts of SP and CGRP (and SS), a property of *drg* neurons (Wood et al., 1990).

In previous experiments *in vivo* (Chapter 3), I have shown that ADX increased SP and CGRP contents of *drg* and that this was reversed by DEX

administration. However, given the complexity of the whole animal, I was not able to demonstrate that glucocorticoids have a direct effect on the *drg*. The experiments described in this chapter confirm and extend my observations *in vivo*. I have now shown that DEX and B both reduce the SP and CGRP contents of dissociated *drg* grown in medium containing NGF. Whilst the mRNA encoding both Type I and Type II receptors has been localised in neurons of the *drg* using *in situ* hybridisation, the action of DEX suggests that steroid action may be mediated by the Type II receptor, although further studies are required to conclusively prove this assertion. In conclusion, I would suggest that glucocorticoids act directly on *drg* neurons to inhibit biosynthesis of SP and CGRP.

CHAPTER 7

**THE DISTRIBUTION OF SP, SS AND CGRP CONTENT OF
DRG FROM LEVEL C1 TO L6 OF THE RAT**

7.1 INTRODUCTION

In previous studies of *drg* from the cervical spinal column (C4-C7, Chapter 3), I have shown that the neuropeptides SP, SS and CGRP are not evenly distributed, but rather, SP and CGRP content was greatest in *drg* from C7 and SS content was greatest in *drg* from C4. The distribution of neuropeptides in sensory ganglia may be of importance in understanding factors regulating the development of neural crest progenitor cells and the functional significance of neuropeptide in sensory ganglia. In the following chapter, a description of the distribution of SP, SS and CGRP in *drg* from C1 to L6 is given.

7.2 METHODS

Six Ham Wistar rats (male, 200g) were killed by asphyxiation in CO₂, the dorsal surface of the spinal column exposed and laminectomy performed to reveal the spinal cord from level C1 to L6 (see Chapter 2). The spinal cord was carefully dissected free, leaving the *drg in situ*, which were removed bilaterally in pairs from each level (C1 to L6) and rapidly frozen on dry ice. After weighing, tissues were homogenised in 2M acetic acid centrifuged at 16000g, for 10 min, and SP, SS and CGRP content of the supernatants determined by RIA (Chapter 2).

7.3 RESULTS

Generally, wet weight (Fig. 7.1a) of *drg* was between 0.3mg and 0.5mg, but this increased in the cervical region between C4 and T1 to a maximum at C7, and in the lumbar region between L3 and L6 to a maximum at L5. The SP content of *drg* (Fig. 7.1b) was found to be distributed similarly to wet weight; minima from C1 to C3 and T4 to T5. In cervical *drg*, SP content increased progressively from C4 to a maximum at C7. In the thoraco-lumbar region, between T8 and L3, SP content was

Figure 7.1

(a) Wet weight (mg) of *drg* from level C1 to L6

(b) Distribution of SP content (pg/ganglion) in *drg* from C1 to L6

Values are mean with S.E.M. to bar

Fig. 7.1a.

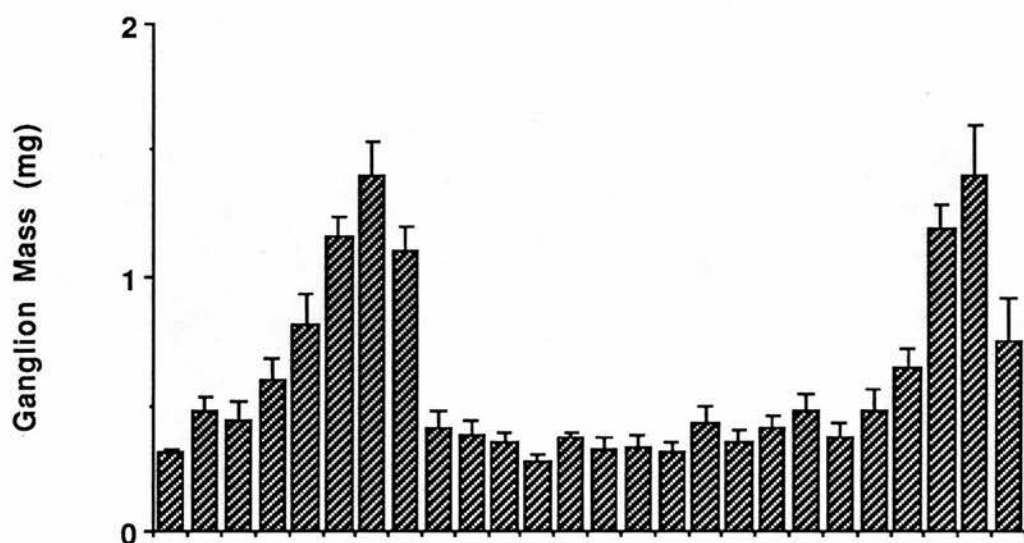
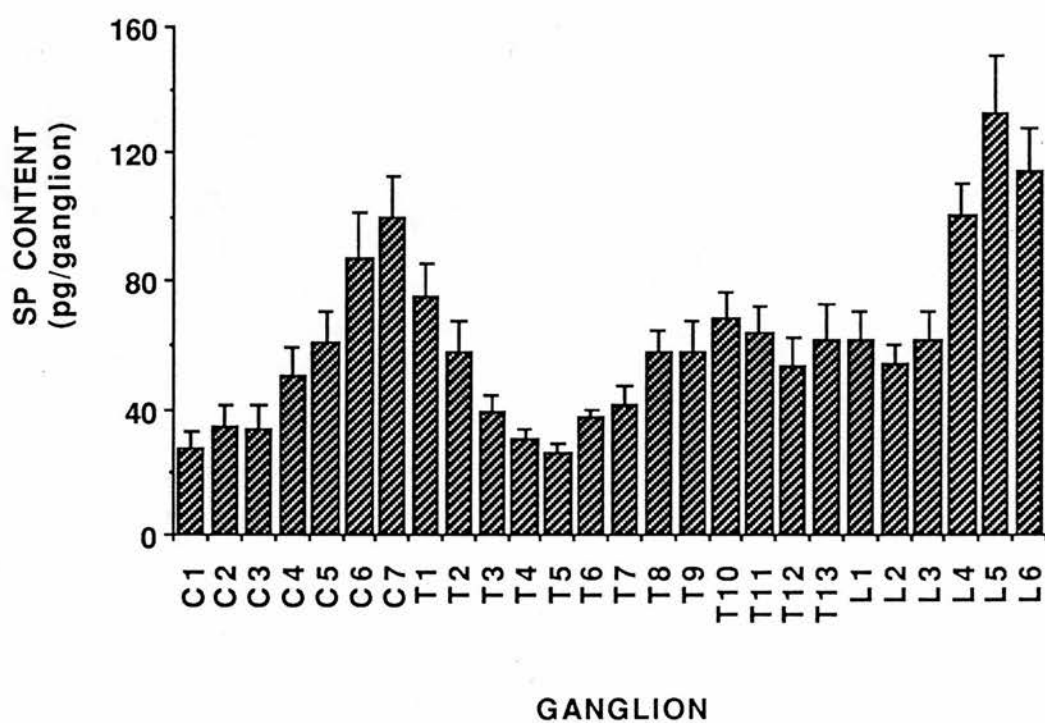


Fig. 7.1b.



relatively uniform. Of the lumbar *drg*, L5 contained the greatest SP. CGRP content of *drg* (Fig. 7.2a) from rostral regions increased moderately from C1 to C7 and subsequently decreased to T5. In thoraco-lumbar regions of the spinal column, *drg* content of CGRP was generally greater than in more rostral *drg*, and maximum in *drg* from L5. SS content (Fig. 7.2b) was distributed in a markedly different pattern from the wet weight, SP and CGRP content. SS content of *drg* from the cervical region of the spinal column was greatest between C2 and C4. Between C4 and L1, SS content was relatively constant and varied between a minimum at C7 and a maximum at T1. In lumbar *drg* from L2 to L6, SS content was greatest in *drg* from L2 and L6.

7.4 DISCUSSION

I have demonstrated, using RIA, that the neuropeptides SP, SS and CGRP were not evenly distributed in *drg* from level C1 to L6. The wet weight of *drg* was relatively uniform, except in the lumbar and cervical enlargements. SP distribution paralleled *drg* wet weight, but CGRP was found to be greatest in *drg* from the more caudal areas of the spinal column, whereas SS content was greatest between C2 and C4 and least in C5, C6 and C7. Large amounts of SS were also found in *drg* from lumbar areas, but elsewhere down the length of the spinal column, SS content was relatively constant.

Little is known of the distribution of neuropeptides in *drg* from different levels of the spinal cord. However, in rat, thoracic *drg* had the highest CGRP content (Gibson et al., 1984b), consistent with this study. Dynorphin content of rabbit *drg* was reported to be relatively uniform down the length of the spinal column (Botticelli, 1981). In the spinal cord a variety of neuropeptides including SP, CGRP (Gibson et al., 1984b), met-enkephalin (Majane et al., 1983), neuropeptide Y (Gibson et al., 1984),

Figure 7.2 (a) CGRP content (pg/ganglion) of *drg* from C1 to L6
(b) SS content (pg/ganglion) of *drg* from C1 to L6
Values are mean with S.E.M. to bar

Fig. 7.2.a

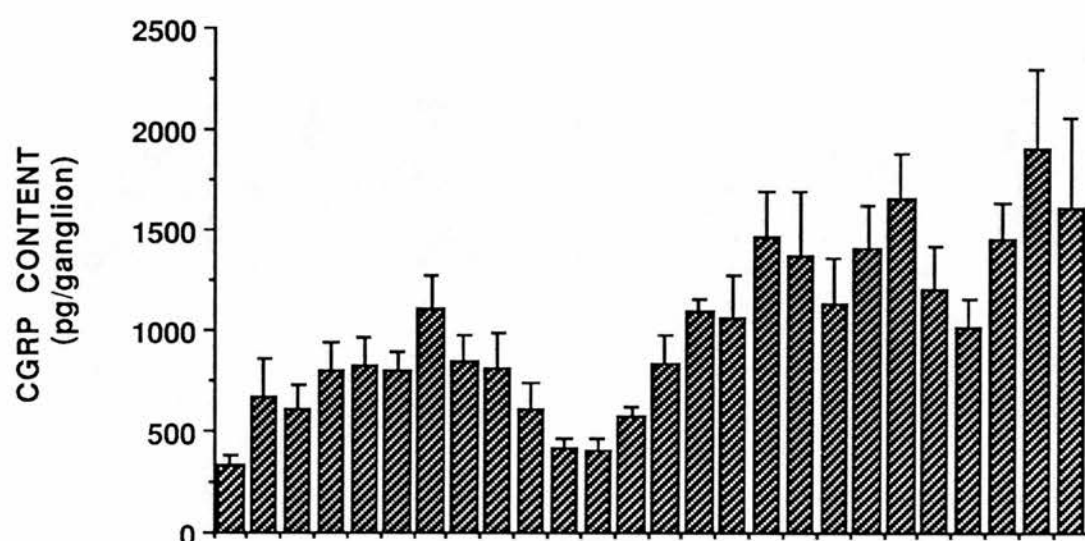
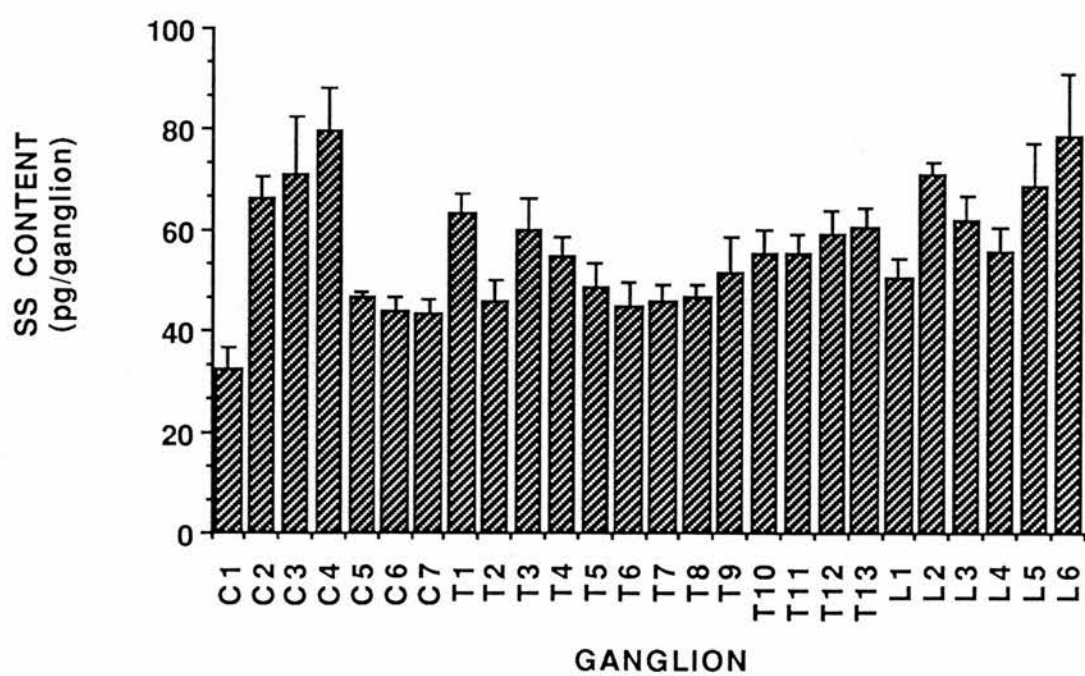


Fig. 7.2.b.



avian pancreatic polypeptide (Hokfelt et al., 1981) and vasoactive intestinal polypeptide (Gibson et al., 1984a; Anand et al., 1983), have been shown to be in highest concentration in lumbo-sacral regions of a number of species (Merighi et al., 1990).

The differential distributions of neuropeptides described here may be a reflection of the cell types found in *drg* from various levels of the spinal column. The neurons of the *drg* can be sub-divided into two histological types based on their staining properties under the light (Hatai, 1902; Lawson et al., 1974) and electron microscope (Duce & Keen, 1977) termed large light or A, and small dark or B, type neurons (for a full description, see Chapter 1); neuropeptides are found predominantly, although not exclusively, in B-type neurons (Ju et al., 1987; Price, 1985, Tuschler & Seybold, 1985; Henken et al., 1988; Hokfelt et al., 1976). In studies of cat *drg*, Rose (1990) has shown that rostral cervical *drg* (C1-C5) have predominantly B type neurons (35-50µm in diameter) whereas 51-77% of perikarya of *drg* from C6 to C8 are A type neurons (more than 50µm in diameter). In thoracic *drg* (Larnicol, 1988), large diameter cells predominate at the T₁ level, but are in the minority at the T₂-T₄ level, where medium diameter cells constitute the major class. The factors that regulate cell diameter of neurons in the *drg* and produce these differences are unknown, but it is likely that this differentiation occurs during development.

Drg arise from progenitor cells of the neural crest and undergo natural cell death during development, where upon the size of the remaining neuronal population of the *drg* is influenced by the extent of the available target tissue ultimately innervated. Thus, in the chick embryo, early removal of a limb bud results in hypoplasia of the corresponding *drg* (Hamburger & Keefe, 1944) and, conversely, transplantation of a supernumerary limb bud causes hyperplasia in adjacent *drg* (Hamburger, 1939; Hamburger & Levi-Montalcini, 1949). In the present study, *drg*

innervating the forelimbs (C4 - C7) and hindlimbs (L4 - L6) had greatest wet weight confirming the relationship between extent of peripheral target and size of *drg*.

The peripheral target not only promotes neuronal cell survival, but may also determine the function of the neurons that innervate that area (Scott, 1986; Frank & Westerfield, 1982; Smith & Frank, 1987; Weiss, 1942; Miner, 1956; Baker & Jacobson, 1970). McMahon (1987) has shown that redirection of nerves innervating skin to muscle reduces the number of SP-containing fibres. Similarly, the number of SP fibres was increased following redirection of muscle afferents to skin. It is thought that target derived neurotrophic factor (NTFs) first postulated by Weiss and others (Weiss, 1942; Sperry & Miner, 1949; Miner, 1956) may mediate the influence of the periphery on *drg*. NTFs are retrogradely transported from the periphery and chronic sciatic nerve section, crush, or inhibition of retrograde transport from the periphery produces biochemical changes in *drg* (Nielsch et al., 1987; Csillick & Knyihar-Csillick, 1982) including changes in neuropeptides. A number of NTFs have been described (Richardson, 1986; Riopelle & Cameron 1987; Richardson & Ebendal, 1982) including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF; Barde et al., 1982) and neurotrophin 3 (NT-3; Hohn et al., 1990). NGF is a heteropentameric polypeptide synthesised in peripheral tissues innervated by sensory neurons (Bandtlow, 1987), it is internalised in nerve terminals and retrogradely transported by peripheral neurons (Palmatier et al., 1984; Korsching & Thoenen, 1983) and administration of NGF protects against the cell death seen following nerve injury or section (Rich et al., 1987; Fitzgerald et al., 1985; Csillick, 1985). *In vitro*, NGF elevates levels of SP and CGRP peptide and mRNA in primary culture of adult *drg* (Lindsay & Harmar, 1989). Neurotrophic factors other than NGF are also present in extracts of peripheral nerve (Richardson & Ebendal,

1983) and a variety of tissue extracts (Lindsay & Tarbitt, 1979; Lindsay & Peters, 1984; Riopelle & Cameron, 1981; Hsu et al., 1984). Brain derived neurotrophic factor (Barde et al., 1982), a peptide extracted from pig brain, has been found to support survival of *drg in vivo* (Kalcheim, 1987) and in culture (Lindsay et al., 1985; Hofer & Barde, 1988). Recently, Nawa & Patterson (1990) have identified a number of factors from heart cell conditioned medium that regulate neurotransmitter choice in sympathetic neurons (Nawa & Sah, 1990). It is probable that a variety of factors released from peripheral and central target areas regulate peptide content of *drg* at different levels of the spinal column.

In conclusion, I have demonstrated that SP, SS and CGRP were not evenly distributed in *drg* down the length of the spinal column. I suggest that one or more NTF may control the development and maintenance of these neuropeptide distributions.

CHAPTER 8

GENERAL DISCUSSION

In this thesis, I have presented results suggesting that adrenal steroids regulate the SP, SS and CGRP content of *drg in vivo*. *In vitro*, glucocorticoids reduced SP and CGRP content of *drg* neurons cultured in the presence of NGF, suggesting that glucocorticoids exert a direct effect upon neuropeptide synthesis in *drg* neurons, possibly mediated by the Type II corticosteroid receptor. Using an adjuvant model of inflammatory disease, I have further demonstrated that CGRP and, possibly SP, in the *drg* may be important in inflammatory processes, and that glucocorticoid regulation of neuropeptides in the *drg* may contribute to their effectiveness as anti-inflammatory agents. In this chapter, I now propose a model of chronic inflammation, in which NGF regulates the involvement of sensory neurons and neuropeptides, and further suggest, that in such a model, glucocorticoids can act at a variety of sites to reduce inflammation and pain.

A key mediator in the development of adjuvant arthritis, and possibly of human rheumatoid arthritis (RA) is thought to be the cytokine interleukin 1 (IL-1; di Giovine & Duff, 1990). IL-1 is present in the synovial fluid of patients suffering RA (Nouri et al., 1984; Marshall et al., 1990) and its concentration is correlated with the severity of inflammation (Rooney et al., 1989; Eastgate et al., 1988). In the synovial fluid, IL-1 is produced by synovial cells, infiltrating leukocytes and activated macrophages (Gery & Waksman, 1972) and chronic infusion of IL-1 enhances development of arthritis in spontaneously arthritic MR/lpr mice (Henderson et al., 1988; Hom, 1990) and development of collagen induced arthritis (Hom, 1988). If administered intra-articularly, IL-1 induces an acute, transient arthritis, induces leukocyte infiltration and loss of articular cartilage similar to that found in adjuvant arthritic rats (Pettipher et al., 1986; Henderson & Pettipher, 1988) and exacerbates development of collagen induced arthritis in mice (Hom, 1988). In culture, it has been found that IL-1 stimulates chondrocytes from healthy articular cartilage to secrete collagen degrading

enzymes (Stephenson et al., 1987), and stimulate synthesis of collagen (Goldring, 1987). Cartilage removed from arthritic joints shows significantly elevated basal release of these enzymes and is more sensitive to IL-1 stimulation (Shinmei, et al., 1988).

When applied to cultured fibroblasts, astrocytes or non-neuronal cells of the sciatic nerve, IL-1 stimulates synthesis of NGF mRNA and peptide and enhances stability of NGF mRNA (Lindholm et al., 1987, 1988; Spranger et al., 1990). NGF is also synthesised in peripheral tissues (Bandtlow et al., 1987; Heumann, 1984), where it is thought to regulate sensory neuron survival in development (see Thoenen & Barde, 1980) and phenotype in adult rats (Fitzgerald et al., 1985). *In vitro* NGF stimulates neurite extension of *drg* neurons (Lindsay, 1988) and as I, and others (Lindsay & Harnmar, 1989), have shown, maintains SP and CGRP mRNA and peptide levels. Weihe (1988) has shown that following induction of adjuvant arthritis, there is an increased ramification of SP and CGRP containing nerve terminals in peripheral tissues and moderately enhanced SP and CGRP content in *drg* innervating the inflamed areas. It may be that during the development of inflammation, IL-1 released from infiltrating leukocytes and macrophages and synovial cells stimulates local enhanced synthesis and release of NGF from the surrounding tissues. The elevated levels of NGF might, in turn, lead to the ramification of nerve fibres within the inflamed tissues and an up-regulation of SP and CGRP content of *drg*.

Thus, in the proposed scheme, sensory neuropeptides, including SP and CGRP, in the *drg*, which are implicated in the pathology of chronic inflammatory states, are up-regulated by NGF released from inflamed tissues. In this model, glucocorticoids might act at one of a number of sites to inhibit inflammatory processes. NGF biosynthesis can be reduced by a direct action of glucocorticoids in a variety of tissues in culture (Siminoski et al., 1986; Wion et al., 1986; Houlgatte et al., 1989; Lindholm et al., 1990),

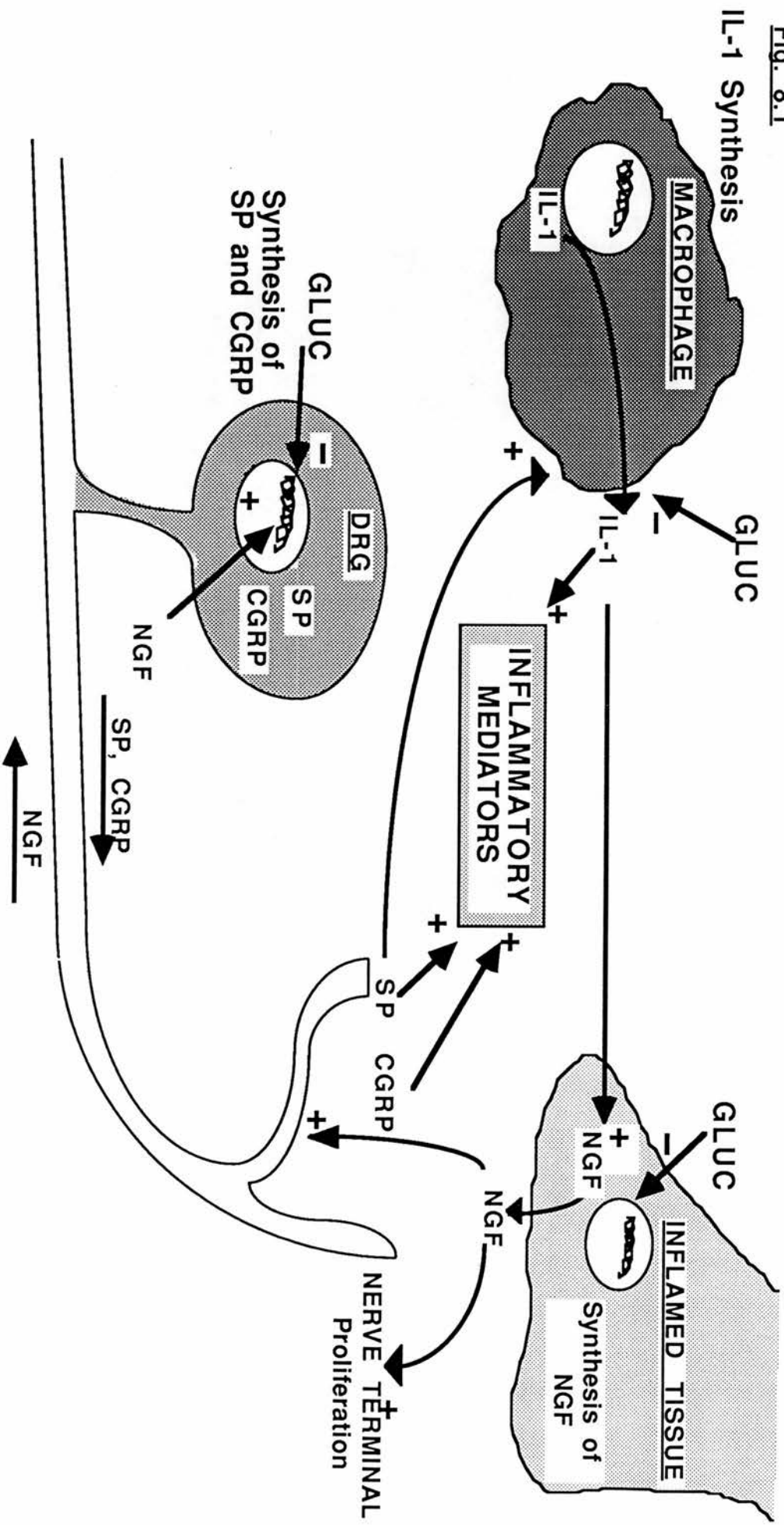
and a similar process may occur in inflamed tissues. In PC12 cells glucocorticoids reduce the levels of NGF receptor which may inhibit the action of NGF (Tocco et al., 1988). Glucocorticoids also inhibit release of IL-1 from macrophages (Snyder & Unanue, 1982), which would reduce IL-1 stimulation of NGF. As a consequence of these actions of glucocorticoids, NGF-mediated up-regulation of SP and CGRP in the *drg* and proliferation of peptidergic terminals in inflamed peripheral tissues would be attenuated. Furthermore, as low doses of SP have been reported to stimulate IL-1 production by monocytes (Lotz et al., 1988; Laurenzi et al., 1990) and P388D1 cells (Kimball et al., 1988), a reduction of SP might act to further reduce IL-1 stimulated synthesis of NGF. Finally, I have shown that glucocorticoids can act directly on the *drg* to reduce both SP and CGRP, resulting in a further reduction in pro-inflammatory drive. The model is summarised in Fig. 8.1.

Thus, whilst glucocorticoid anti-inflammatory activity is undoubtedly complex and varied (see Chapter 1), it is possible that some of the anti-inflammatory activity is mediated by an inhibition of the processes elucidated above. In conclusion, I have demonstrated that in a rat model of inflammatory disease, the neuropeptides CGRP, and possibly SP, are increased in the *drg*. Furthermore, I have shown that glucocorticoids reduce the inflammation and attenuate the increase in CGRP content. In intact animals, I have shown that glucocorticoids also reduce SP and CGRP content, but increase SS content of *drg*. *In vitro*, I have shown that this is a direct effect of glucocorticoids on the *drg*, probably mediated via the Type II receptor. Finally, I have suggested that glucocorticoids may produce some of their anti-inflammatory effects through direct and indirect actions on the neuropeptides in *drg*.

Figure 8.1

Schematic diagram of a possible model for the involvement of sensory neurons and neuropeptides in the development of inflammatory pathologies. The diagram also shows sites at which glucocorticoids (GLUC) might act to inhibit this process and thereby produce their anti-inflammatory effects

Fig. 8.1



APPENDIX

Composition of Ham's F-14 (without Sodium Bicarbonate and antibiotics)

Constituents	mg/l
Amino Acids	
L-Alanine	17.70
L-Arginine HCl	422.00
L-Asparagine H ₂ O	30.01
L-Aspartic Acid	26.50
L-Cysteine HCl.H ₂ O	70.32
L-Glutamic Acid	29.30
L-Glutamine	292.00
Glycine	14.90
L-Histidine HCl.H ₂ O	41.96
L-IsoLeucine	7.94
L-Leucine	26.10
L-Lysine HCl	72.90
L-Methionine	8.88
L-Phenylalanine	9.96
L-Proline	68.90
L-Serine	20.90
L-Threonine	23.90
L-Tryptophan	4.04
L-Tyrosine	15.58
L-Valine	23.30

Constituents	mg/l
Vitamins	
d-Biotin	0.0073
D-Ca Pantothenate	0.48
Choline Chloride	13.96
Folic Acid	1.30
i-Inositol	18.00
Nicotinamide	0.037
Pyridoxine HCl	0.062
Riboflavin	0.038
Thiamin HCl	0.340
Vitamin B12	1.36
Ascorbic Acid	15.00
Inorganic Salts	
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	283.22
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.00249
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.83400
KCl	372.80
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	174.00
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	37.00
NaCl	7599.00
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	268.00
Other Components	
D-Glucose	1981.80
Hypoxanthine	4.00
Linoleic Acid	0.084

Constituents	mg/l
Lipoic Acid	0.210
Phenol Red Na ₂	1.20
Putrescine 2HCl	0.1610
Sodium Pyruvate	220.00
Thymidine	0.730

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